A dielectric barrier discharge plasma degrades proteins to peptide fragments cleaving the peptide bond

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Abstract: Degradation of proteins by atmospheric pressure plasmas was described in the literature several times. However, the degradation products have not been characterized in detail. Using different methods we confirmed that degradation takes place. We showed that proteins are cleaved at the peptide bonds resulting in accumulation of peptides and amino acids. Using D-mannitol as scavenger, the importance of plasma-generated hydroxyl radicals was demonstrated.

Keywords: hydroxyl radicals, superoxide, protein carbonylation, amino acids

1. Introduction

The effect of atmospheric pressure plasmas on proteins is of special interest in the field of plasma medicine. On the one hand, proteins are the most abundant biomolecules in cells and also highly abundant in body fluids. Plasma exposure of cells and other biological samples therefore involves the exposure of proteins. The inactivation of bacterial cells has been shown to correlate with protein inactivation [1]. On the other hand, proteinaceous contaminations can pose a serious health risk, for instance when surgical instruments are contaminated with prions - proteins that can cause diseases [2]. It is desirable to inactivate such contaminations and to understand the inactivation mechanism. To study the inactivation and removal of proteins, frequently model proteins are applied, which are either dried on surfaces to simulate surface contaminations or treated as aqueous samples. Upon plasma treatment, modifications of amino acid side chains were observed, which can cause protein inactivation [1,3]. Additionally, protein etching and degradation have been described in the literature [4-8]. To our knowledge, however, the products and the mechanism of plasma-mediated protein degradation are still unknown.

For some of the clinical application of plasma it is crucial to identify the degradation products. Prions, for instance, are neurodegenerative infectious proteins. Their removal *e.g.* from surgical instruments is part of latest research in plasma medicine [4]. Some fragments of prions have been shown to remain infectious [9,10]. An accumulation of potentially infectious prion fragments should be avoided to ensure the safety of plasma decontamination.

All proteins are built from the same building blocks, the proteinaceous amino acids, that are linked by peptide bonds (also referred to as amide bonds). Since the degradation of proteins by plasma was observed independent from the primary protein structure (amino acid sequence), we hypothesized that plasma causes the cleavage of peptide bonds leading to fragmentation of proteins into smaller peptides. We applied different assays for quantification and visualization of protein fragments to test this hypothesis and to identify the degradation products.

2. Results and discussion

Bovine serum albumin (BSA) is a model protein commonly used to analyze the impact of plasma on proteins. We treated BSA (40 µl of a 1 mg ml^{-1} aqueous solution) with a dielectric barrier discharge plasma (DBD, Cinogy, Duderstadt, Germany) for up to 10 min and measured the protein concentration using three methods: colorimetrically with the Bradford assay, direct absorption measurement at 280 nm, and SDS polyacrylamide gel electrophoresis (SDS-PAGE) (**Fig. 1A, B**). In the Bradford assay the decrease in protein amounts was observed and SDS-PAGE analysis confirmed a reduction in the amount of full length protein. When determining the protein concentration by measuring the absorption at 280 nm and applying Lambert-Beers' law, the protein concentration seemed to increase from initially 1 mg ml^{-1} up to 1.7 mg ml^{-1} after 10 min plasma treatment (**Fig. 1A**). A closer look at the absorption spectra revealed that the peak at 280 nm typically used for protein quantitation was buried under signals of unknown origin (**Fig. 1C**). Many plasma-generated species absorb in the near-UV range [11] and thus might mask the protein-based peak at 280 nm. The determination of protein concentrations by absorption measurements is a common method, our data however indicate that when dealing with plasma-treated solutions, special care has to be taken to avoid interferences by plasma-derived species.

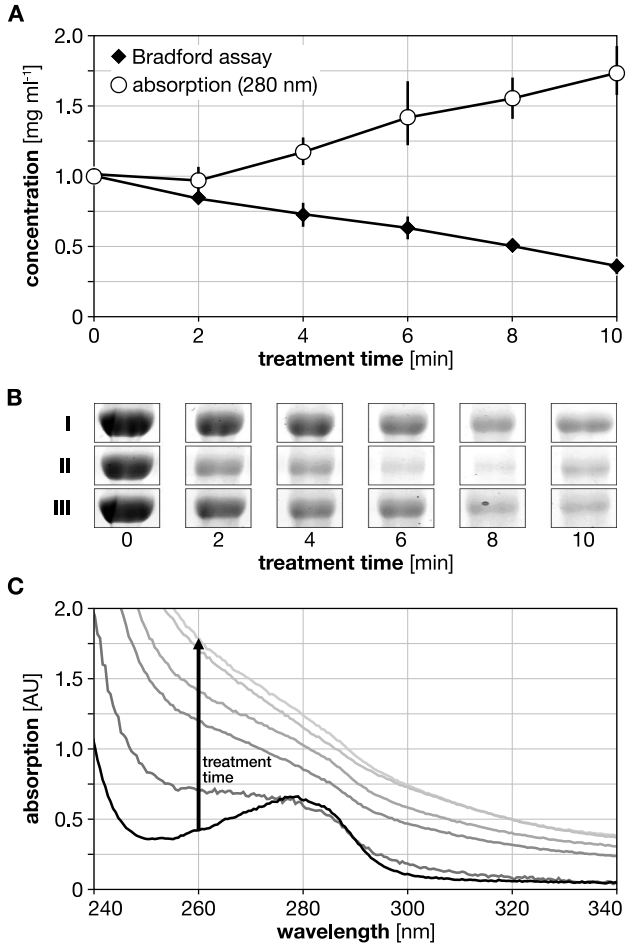

Fig. 1. Quantitation and detection of BSA after plasma treatment. (A) Quantitation using the Bradford assay or the absorption at 280 nm in conjunction with applying Lambert-Beers' law. (B) Detection of BSA after plasma exposure and protein separation by SDS-PAGE. The results of three independent replicates (I-III) are shown. (C) Absorption spectra of plasma-treated BSA (0, 2, 4, 6, 8, and 10 min).

As stated above, we hypothesized that proteins are degraded by cleavage of peptide bonds due to plasma-derived species. If true, as a result of the reaction one would expect the generation of peptides or free amino acids and a corresponding increase in the amount of N- and C-termini. Ninhydrin is a compound known to react with amino groups ($-NH_2$) resulting in the formation of a dye called Ruheman's Purple [12]. BSA was exposed to DBD plasma and the samples analyzed using a ninhydrin-based assay for quantification of terminal amino groups (**Fig. 2A**). Indeed, the number of terminal amino groups increased treatment time dependently.

Acidic hydrolysis is a commonly used method for complete degradation of proteins into amino acids for instance prior to amino acid analysis by HPLC. A 60 minute treatment of BSA with the DBD plasma resulted in the same concentration of terminal amino acids as acidic hydrolysis (about 2.2 mM), although the curve shape indicates that even higher concentrations might be possible by prolonging plasma treatment time (**Fig. 2A**). To test if other proteins are degraded in a similar fashion, degradation kinetics of four other proteins were obtained and benchmarked with acidic hydrolysis: lysozyme, ribonuclease A (RNase A), superoxide dismutase A (SodA), and SodB (**Fig. 2B-E**). For all tested proteins, increased amounts of terminal amino groups were observed, showing that peptide bond cleavage is not a protein-specific degradation mechanism.

In the interaction of plasmas with water in air a variety of reactive oxygen and nitrogen species are generated that differ in reactivity and lifetime [13]. To find out, whether the reactive species causing peptide bond cleavage is short or long-living, we incubated plasma-treated BSA at room temperature for up to 1 h and compared the concentration of terminal amino groups to samples subjected to the ninhydrin assay directly after treatment. In both cases the same concentration of terminal amino groups was observed, excluding long-living species like H_2O_2 or peroxynitrite from being involved in peptide bond cleavage.

Next, we investigated the effect of superoxide and hydroxyl radicals as short-living species. Superoxide dismutases are highly efficient scavengers of superoxide, yet treatment of the two superoxide dismutases SodA and SodB resulted in their fragmentation (**Fig. 2D, E**). O_2^- therefore is likely not the primary species causing peptide bond cleavage.

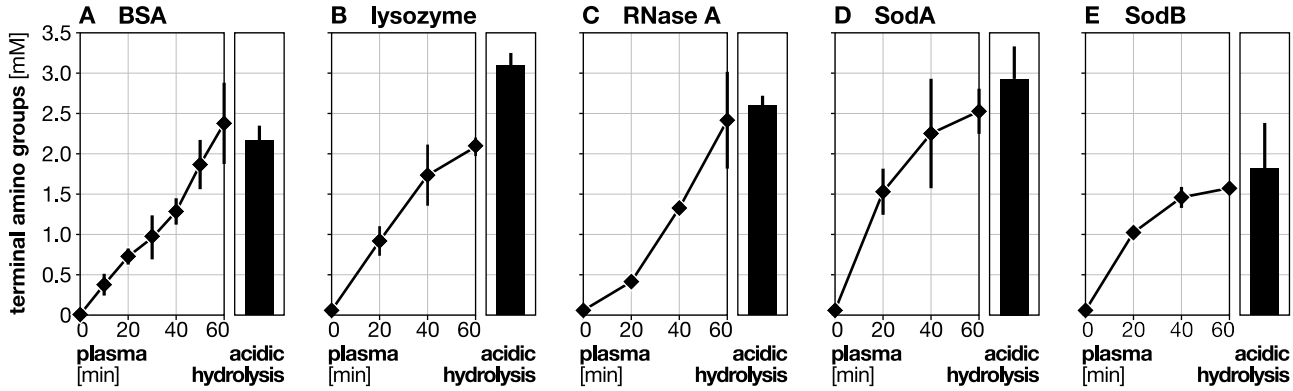

Fig. 2. Quantification of terminal amino groups in protein solutions after plasma exposure or after acidic hydrolysis using the ninhydrin assay.

When 10 mM D-mannitol, a potent •OH scavenger, were added to BSA prior to plasma treatment, the number of detected terminal amino groups was reduced from 570 µM to 350 µM (**Fig. 3**). Further increasing the concentration of D-mannitol (100 mM) did not provide additional protection. Since •OH scavenging by D-mannitol did not completely prevent protein cleavage, another plasma-generated species might also contribute to protein degradation, like for instance singlet oxygen or atomic oxygen.

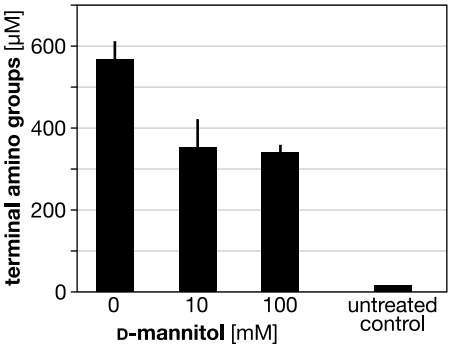

Fig. 3. Quantitation of terminal amino groups after exposure of BSA to DBD plasma for 15 min. D-mannitol was added prior plasma treatment as •OH scavenger.

In the literature the degradation of proteins by reactive oxygen species has been described previously and a mechanism for peptide bond cleavage was postulated (**Fig. 4**) [14-16]. In this model, hydroxyl radicals attack the αC atom engaged in the peptide bond and abstract a hydrogen atom resulting in a carbon radical. After this initial step, several reactions with molecular oxygen and peroxyl radicals ($HO_2^•$) take place. As final products, two fragments are formed, one exhibiting an N-terminal amino group, the other a carbonylated C-terminus. Since the latter has a diamide-like structure, this mechanism was termed diamide pathway of protein fragmentation [16].

Based on our results, the plasma-mediated protein degradation shares some attributes with the diamide pathway, namely an involvement of hydroxyl radicals. Whether or not the plasma-mediated degradation also involves peroxyl radicals is not entirely clear yet. Peroxyl radicals should be removed effectively by superoxide dismutases, however, it remains to be tested if superoxide dismutases remain active when treated with plasma.

The characterization of protein degradation products provides important insights into plasma-based sterilization and deepens our understanding of plasma-protein interactions.

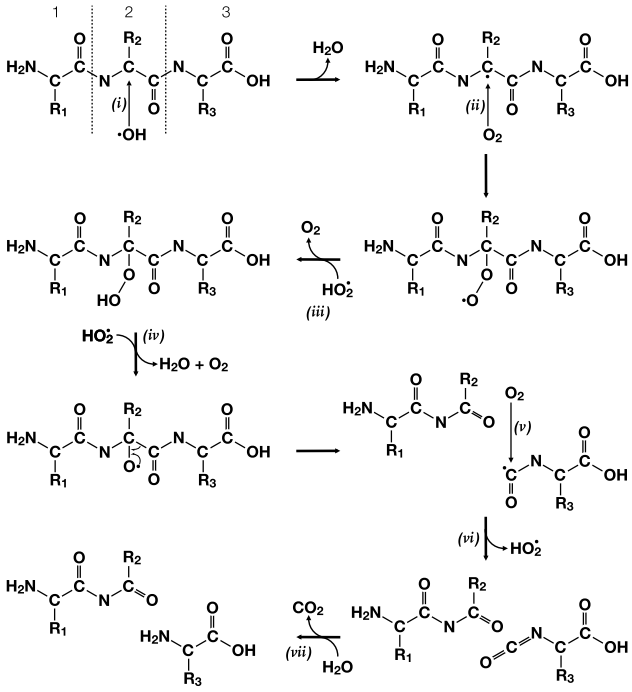

Fig. 4. Postulated mechanism for cleavage of peptide bonds by reactive oxygen species. Redrawn based on [14-16].

3. References

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