Spin-trapping agent α-phenyl *N*-tert-butylnitrone binds to trypsin and enhances heparin-induced inhibition of amidolytic activity and structural degradation of the enzyme

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Abstract The effects of heparin on trypsin have recently been demonstrated to involve inhibition of catalytic activity and degradation of the enzyme by means of an oxidative mechanism. The possibility that α-phenyl *N*-tert-butylnitrone protects heparin-induced radical formation on trypsin was investigated by measuring amidolytic activity and changes in the structure of trypsin in the presence of heparin with and without α-phenyl *N*-tert-butylnitrone. The results show that α-phenyl *N*-tert-butylnitrone does not only prevent, but it even significantly enhances effects of heparin on the enzyme. This is due to the unique property of α-phenyl *N*-tert-butylnitrone, independently of spin-trapping capacity, to modify the trypsin structure by binding irreversibly to the catalytic triad, at sites distinct from those to which heparin binds.

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Key words: α-Phenyl *N*-tert-butylnitrone; Heparin; Trypsin; Bovine; Enzyme inhibition; Oxidation

1. Introduction

In recent years, many scientific reports have dealt with the role of various reactive oxygen species in the etiology of inflammatory and degenerative pathologies affecting various tissues and organs. A general consensus is emerging that the generation of free radicals and consequent oxidative stress represent the molecular mechanism underlying both age-dependent degenerative processes [1] and more severe conditions such as stroke, Alzheimer's disease, diabetes and cancer [1–5]. Much effort has thus been spent in order to characterize the nature and extent of the oxidative process occurring in different conditions and to identify possible anti-oxidative agents to be tested both in vitro and in vivo for ameliorating oxidative damage. Among the various anti-oxidants tested [2–4,6–9], spin-trapping agents such as α-phenyl *N*-tert-butylnitrone (PBN) have been demonstrated to be effective both in in vitro systems and in experimental in vivo models in reducing and preventing tissue alterations due to radical-induced oxidative stress [4,6,9].

We recently demonstrated that heparin, a highly negatively charged glycosaminoglycan employed as anti-thrombotic and anti-coagulative agent in clinical practice, binds to various proteins in a rapid and specific manner followed by significant structural and functional alterations of the protein depending on its nature [10–12]. This specific binding has also been demonstrated to trigger oxidative reactions responsible for the most significant oxidative damage on proteins [10,11,13], particularly evident when trypsin is used as reference protein [10,13]. The oxidative nature of the process observed in the trypsin-heparin interaction has been further confirmed in recent studies by the presence of radicals in the EPR spectra of trypsin with heparin using PBN as spin-trapping agent [13]. This finding prompted us to test the possibility that the oxidative effects of heparin are antagonized by radical spin-trapping, so that the trypsin function and structure should be preserved. For this purpose, the effect of PBN, in absence and presence of heparin, was tested on bovine trypsin by measuring the structural and functional properties of the enzyme in various conditions of incubation and time of addition of either one or both substances. Unexpectedly, results indicated that not only does PBN not antagonize or prevent heparin effects on trypsin, but it enhances heparin-induced enzyme inactivation and degradation.

2. Materials and methods

Trypsin (EC 3.4.21.4, TPCK-treated, from bovine pancreas), *N*α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA), PBN and soybean trypsin inhibitor (STI) were from Sigma. Low molecular mass heparin (5.2 kDa) from pig intestinal mucosa, with about nine disaccharide units/molecule, was a generous gift of Prof. B. Casu, 'G. Ronzoni' Institute of Chemistry and Biochemistry, Milan. Its purity was assessed as reported previously [14]. All other chemicals were of the highest available grade.

Solutions were made up in purified distilled water and 20 mM Na-phosphate buffer (pH 6.0) with 0.15 M NaCl (PBS) filtered through 0.2 μm Millipore filters. Electrospray mass analysis (ES-MS) showed that trypsin contained only one active species. Trypsin was used at a final concentration of 0.75 mg/ml (32 μM), in the absence and presence of 25 mM PBN, with and without heparin at 36 and 360 μg/ml (6.9 and 69 μM, respectively). Solutions were tested both fresh and after incubation for 2.5 h at 37°C in a stirring bath.

Circular dichroism (CD) spectra were measured on a Jasco J-715 spectropolarimeter. Solutions of PBN (25 mM), in the absence and presence of trypsin after incubation at 37°C for 2.5 h, were analyzed in a quartz cell of 0.1 cm path length and spectra were recorded in the wavelength range of 400–320 nm. This range excluded wavelengths at which both PBN and amino acid residues of the proteinase absorbed, thus permitting measurement of any dichroism signal generated by the carbon atom of PBN which had become chiral in the presence of trypsin. Four scan spectra were recorded using a scanning velocity of 10 nm/min. Air and water baseline values were subtracted from each spectrum.

EPR spectra were measured at room temperature using a Bruker

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Abbreviations: PBN, α-phenyl *N*-tert-butylnitrone; BAPNA, *N*α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride; STI, soybean trypsin inhibitor

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ESP 300 X-band spectrometer. Samples were analyzed in a flat quartz cell to minimize dielectric dispersion of aqueous solvent. A modulation field of 100 kHz was used with an amplitude of 1 Gauss.

The activity of trypsin in the absence (control) and presence of heparin and PBN, both alone and together, was measured at 410 nm on a Shimadzu UV-160 visible recording spectrophotometer as the concentration of *p*-nitroaniline forming by degradation of BAPNA at 25°C in 50 mM Tris-HCl with 20 mM $CaCl_2$, pH 8.3 [15]. 10 μl aliquots of trypsin (128 nM, final concentration), both fresh (in progress curve experiments) and incubated (drawn at 0.5 h intervals over a period of 2.5 h), in the absence (control) and presence of PBN and heparin, were added to the substrate (0.6 mM BAPNA) in thermostated cuvettes at 25°C. Reactions were followed by monitoring the increase in absorbance every minute for 15 min. The reaction was found to be linear up to 30 min. In separate progress curve experiments, PBN and heparin were added to the substrate in the assay buffer and the reaction was started by the addition of trypsin. In control experiments, it was confirmed that neither heparin nor PBN alone had any proteolytic activity on the substrate.

SDS-PAGE was performed according to the method of Laemmli [16] on a 18% acrylamide gel by loading 25 μg proteins of each sample per lane without any prior heating or reducing treatment. Gels were stained with Coomassie brilliant blue. In non-denaturing electrophoresis, SDS was replaced by Triton X-100 in the running and sample buffers at final concentrations of 10 (v/v) and 5%, respectively. Sample proteins were 50 μg/lane.

ES-MS measurements were carried out at the Glaxo Research Laboratories in Verona (Italy), using a Biotech single quadrupole VG-Platform instrument. Analysis was performed on 300 μg proteins of trypsin incubated with PBN in the absence and presence of heparin, lyophilized and redissolved in water containing 1% acetic acid. 25–50 pmol/μl of each sample was directly injected into the ES source using a Phoenix 20 CU pump, at a flow rate of 10 μl/min. The acceleration potential was 37 V at a source temperature of 40°C. The scanning range was 550–120 m/z.

Statistical analyses were performed by GraphPad PRISM (version 2.0). The influence of the variables (PBN and heparin) on trypsin activity was tested by two-way analysis of variance (ANOVA) and the means between groups by non-parametric tests.

3. Results and discussion

In preliminary experiments, the effects of increasing PBN concentrations (5, 10, 15 and 25 mM) tested on trypsin after incubation at 37°C for 2.5 h gave activity inhibitions of 12, 21, 25 and 30%, respectively. The characteristics of PBN binding to trypsin were investigated by following the velocity of the reaction in the first 15 min, after either enzyme or substrate was added to the incubation mixture to start the reaction. This procedure can discriminate between the reversibility and irreversibility of enzyme activity inhibition [17]. When PBN was added to the substrate before enzyme addition, the velocity did not change and the curves in the absence and presence of PBN overlapped (data not shown). When PBN was added to the enzyme before substrate addition, the velocity decreased (Fig. 1A: slopes of curves in absence and presence of PBN, 0.0181 and 0.0157, respectively, $P < 0.05$), indicating irreversible inhibition of the enzyme [17]. A rapid and irreversible enzyme inhibition was also caused by heparin alone. This effect was further enhanced by PBN, reaching statistical significance at the lowest heparin concentration (Fig. 1A: slope of curve, 0.0146, $P < 0.05$ for comparisons with trypsin controls without and with 36 μg/ml heparin).

As shown in Fig. 1B, at each heparin concentration, PBN was able to enhance the heparin-induced, time-dependent inhibition of enzyme activity. There was a significant individual effect of PBN and heparin on trypsin activity without any interaction between the two substances (two-way ANOVA,

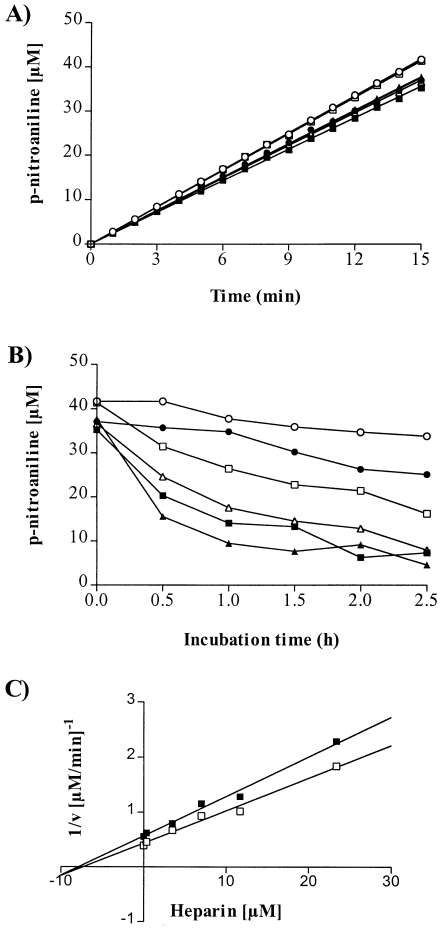

Fig. 1. Amidolytic activity of trypsin. Effects of separate and combined addition of heparin and PBN. (A) Progress curves of activity in the first 15 min reaction with fresh reactants. (B) At each time, values of activity after 15 min reaction are reported. Each point is the mean of three measurements made on separate occasions. Both in (A) and (B), activity without PBN and with PBN is indicated by empty and filled symbols, respectively. Control trypsin: -○-, -●-; trypsin with heparin at 36 μg/ml (□, ■) and 360 μg/ml (△, ▲). (C) Dixon plots of 1/v of enzyme activity versus different heparin concentrations in the absence (□) and presence of a fixed concentration (25 mM) of PBN (■).

$P = 0.0229$ and $P < 0.0001$ for PBN and heparin effects, respectively). Analysis of enzyme activity made by Dixon plots [19] with various heparin concentrations in the absence and presence of a fixed PBN concentration (25 mM) (Fig. 1C) did confirm that PBN and heparin behave as non-competitive inhibitors of trypsin, causing synergistic inhibition due to binding to different sites on the enzyme [18].

In the presence of PBN alone, the electrophoretic pattern of trypsin turned out to be significantly altered in SDS-PAGE (Fig. 2A, lane 2 versus lane 1), with disappearance of the main trypsin band of about 24 kDa. When PBN was used together with heparin, the dose-dependent pattern of trypsin fragmentation due to heparin alone (lanes 3 and 5) was further worsened (lanes 4 and 6). In order to exclude the possible effect of SDS in causing the structural modifications of trypsin observed with PBN, with and without heparin, non-denaturing electrophoresis was performed on the same samples (Fig. 2B). While PBN alone caused a significant change of the band closest to the cathode, which almost completely disappeared

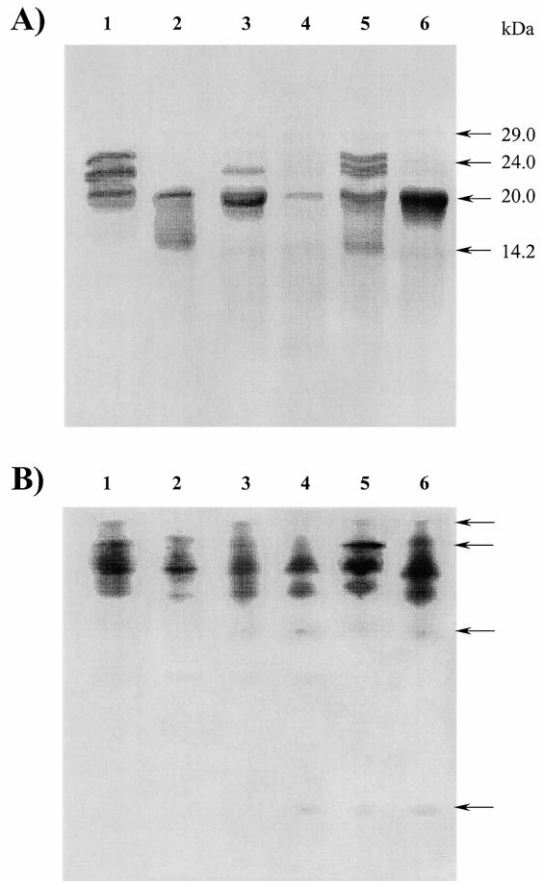

Fig. 2. SDS- and non-denaturing PAGEs of trypsin in the absence and presence of heparin with and without PBN after incubation for 2.5 h at 37°C. Lanes in (A) (SDS-PAGE) and (B) (non-denaturing electrophoresis) are 1, trypsin; 2, with PBN; 3 and 4, with 36 μg/ml heparin, without and with PBN; 5 and 6, with 360 μg/ml heparin, without and with PBN. (B) Cathode at the top.

(lane 2), the addition of heparin gave rise to a marked reduction in another cathodal band, together with the appearance of a new band closer to the anode (Fig. 2, arrows on the right) which was due to the effect of heparin alone on trypsin.

ES-MS analysis confirmed that trypsin underwent almost complete fragmentation when PBN and heparin were added together (Fig. 3). The 23 320 Da species referring to intact trypsin, the only one visible in trypsin samples with PBN alone (1), could no longer be detected in samples with PBN and heparin, but was replaced by various fragments of a lower molecular mass (2 and 3). At the highest heparin concentration, more numerous trypsin fragments were present and they also differed in molecular mass from those seen at the lowest heparin concentration. This result fits those of amidolytic activity and electrophoretic data showing massive indiscriminate fragmentation of the proteinase which loses activity. Mass analysis results were strikingly different from those reported previously on trypsin in the presence of heparin alone [13], which showed that trypsin underwent more limited fragmentation with the appearance of only two trypsin fragments in addition of the intact trypsin species.

STI was used as a tool to explore the nature of the heparin plus PBN interaction with the catalytic site of trypsin. If heparin effects were mediated by its binding to sites involving the catalytic triad of trypsin, as previously proposed [10], and if this property was also shared with PBN, then, prior inhibition of the catalytic site by a known specific inhibitor would be expected to prevent or reduce heparin effects, with and without PBN, i.e. trypsin would undergo less or no degradation at all. In order to test this hypothesis, STI was used at both non-saturating (0.25/1, STI/trypsin weight ratio) and saturating (1/1) concentrations and the formation of the 43 kDa inhibitory complex was followed in SDS-PAGE. At weight ratios with trypsin ranging from 0.25 to 1.0, STI caused a rapid, dose-dependent inhibition of trypsin, consistent with the known standard mechanism of action of this serine proteinase inhibitor [19] (data not shown). Thus, the maximal inhibitions of trypsin expected to occur at each STI concentration were observed just after the addition of the inhibitor and did not change after incubation for 2.5 h at 37°C. In separate experiments, it was also verified that neither PBN nor heparin alone interacted with STI.

STI at a 0.25/1 weight ratio with trypsin caused a mean ± S.D. enzyme inhibition of 38.8% ± 5.8 ($n = 6$). The addition of PBN increased the enzyme inhibition by a value equivalent to the inhibition produced by PBN alone. This result indicates that PBN binds to free catalytic sites on trypsin without interfering with STI binding. The fluorescence emission spectra and SDS-PAGE of STI with heparin confirmed that there was no interaction between these two substances. Also, incubation of STI with heparin for 2.5 h at 37°C did not alter the inhibitory capacity of STI either (data not shown). Thus, any modification of trypsin seen after the addition of heparin and PBN in the presence of STI would be expected to be due to the interaction of heparin and PBN with trypsin only. The effects of PBN together with heparin at both saturating and non-saturating concentrations of STI were evaluated by means of SDS- and non-denaturing electrophoresis, since the significant absorbance of PBN even at the wavelengths of 350–320 nm prevented any spectroscopic analysis of structural modifications of trypsin. Densitometric analysis of the band of the trypsin-STI complex at low STI concentrations showed that it was more intense in the presence of PBN than in its absence (Fig. 4, lane 3 versus lane 1), consistent with data on the amidolytic activity of trypsin showing that PBN has an additive effect on inhibition by STI. When the STI/trypsin complex was maximal (i.e. at a 1/1 ratio: activity inhibition of 96%), the addition of PBN with heparin at both concentrations no longer caused the fragmentation of trypsin seen in the absence of STI (lanes 4 and 6 in Fig. 2A versus lanes 6 and 8 in Fig. 4). At non-saturating STI concentrations, which may still allow heparin to bind to free sites on trypsin, the trypsin bands still had a slightly higher mobility and staining was more diffuse in the low molecular mass region, consistent with the occurrence of limited damage to the enzyme structure (Fig. 4, lanes 5 and 7). Thus, heparin binding to the catalytic site of trypsin is necessary in order for heparin to cause enzyme fragmentation sustained by the generation of radicals. The EPR spectra of trypsin with heparin in the presence of STI (at any concentration) confirmed that signals indicating radical formation could no longer be detected (data not shown).

In order to verify whether the carbon atom of the PBN molecule was involved in binding to trypsin, the CD spectra of trypsin with PBN were performed in the wavelength region beyond that of absorbance of amino acid residues. The result-

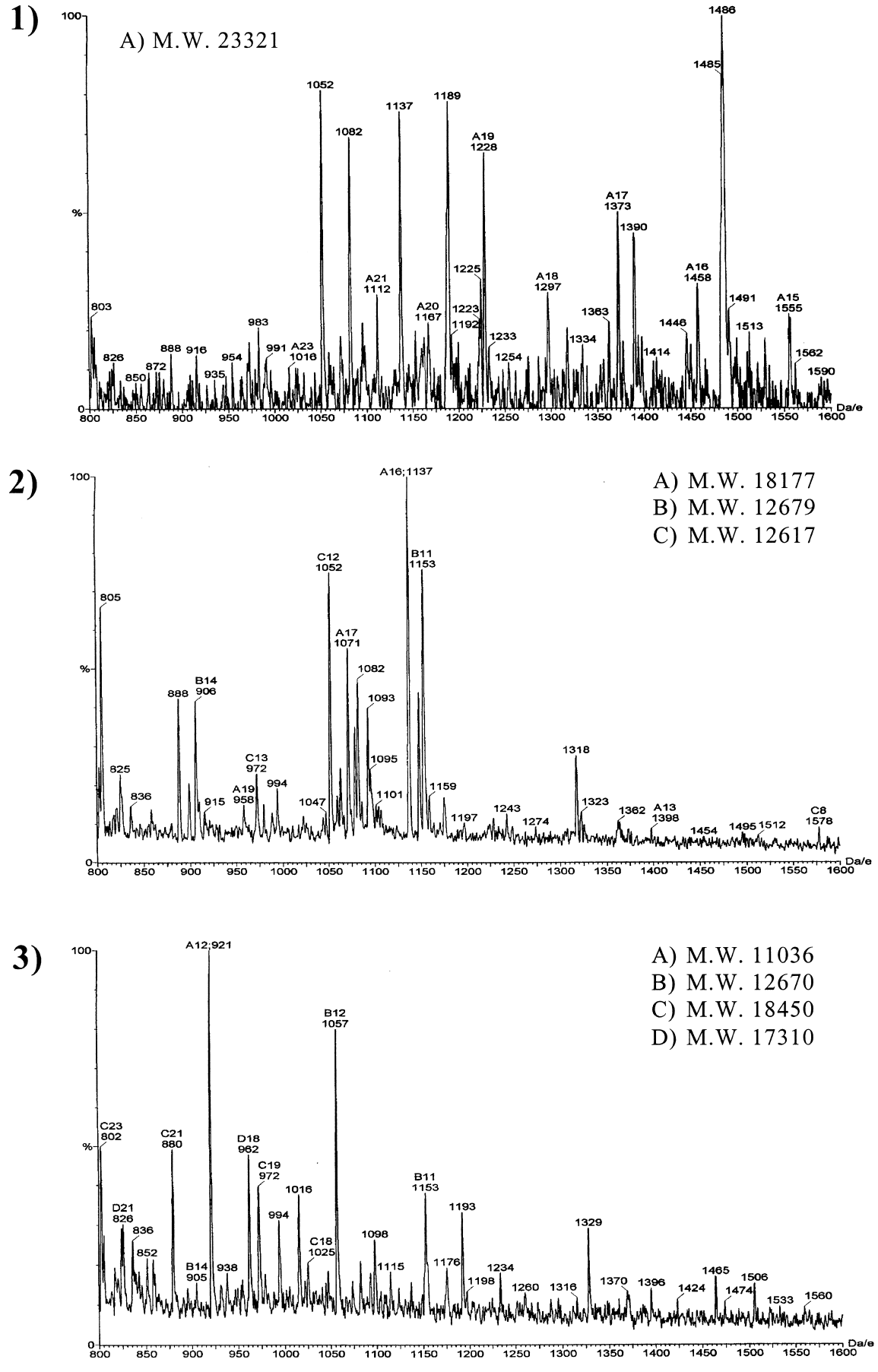
1)
A) M.W. 23321
2)
A) M.W. 18177
B) M.W. 12679
C) M.W. 12617
3)
A) M.W. 11036
B) M.W. 12670
C) M.W. 18450
D) M.W. 17310
Da/e

Fig. 3. Electrospray mass spectrometry analysis of trypsin incubated with PBN in absence and presence of heparin. Analysis was performed as specified in Section 2 on 300 μg proteins of trypsin incubated with PBN in absence (control, 1) and presence of 36 μg/ml (2) and 360 μg/ml heparin (3). Spectra are presented with the corresponding molecular mass of peaks detected.
←

ing signal did not differ at all from control signals, thus negating the possibility that the carbon became chiral because of its binding to the proteinase.

4. Concluding remarks

The results of this work reveal an unexpected, apparently paradoxical effect of PBN, capable to enhance the heparin-catalyzed oxidative degradation of trypsin. This effect is due to the unique property of PBN to bind irreversibly to sites on the catalytic triad, altering the enzyme structure in such a way that binding of heparin and the following radical production is further increased.

The results also suggest that N-linked oxygen on the PBN molecule is the most likely reactive site involved in binding. The negatively charged oxygen may either take ion linkages with the positively charged N-atom of a histidine residue (His_{57}) or drive a nucleophilic attack on the β-carboxyl group of an aspartic acid residue (Asp_{102}) of the catalytic triad in trypsin, whereas the carbon atom of PBN would be free to trap the radical, as expected. Thus, the effect of PBN in enhancing radical-dependent effects of heparin is independent of the spin-trapping property, which is apparently preserved (if this were not the case, no EPR spectra would ever been seen with trypsin plus heparin [13]). Considering that radicals generated by heparin are mostly protein-bound [13], PBN is expected to exert its spin-trapping property only when it is bound to trypsin, blocking radicals which form in close proximity to its binding site.

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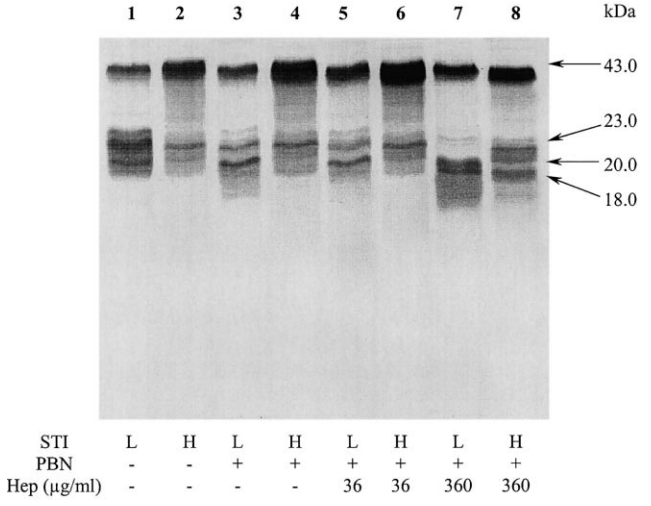

Fig. 4. SDS-PAGE of trypsin complexed with STI. Effects of PBN and PBN plus heparin after incubation for 2.5 h at 37°C. STI was added to trypsin immediately before the addition of PBN and heparin. L, H: low and high concentrations of STI (0.25/1 and 1/1 STI/trypsin weight ratios).

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