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Peptide damage under Fenton conditions is sequence-dependent

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Peptides with two or more acidic amino acids are damaged to a greater extent than other peptides under Fenton conditions as revealed by treating a 29791 membered one-bead-one-compound peptide library with $FeCl_3$, sodium ascorbate and hydrogen peroxide.

Radical mediated protein damage is thought to play an important role in several diseases and ageing.[1,2] Often radicals are generated by electron transfer from a metal ion like Fe^{2+} to O_2 or H_2O_2 (Fenton reaction) and numerous studies revealed that the damage occurs through oxidation of the amino acid side chains and backbone cleavage.[2] In contrast, little is known as to whether some peptides are cleaved to a higher extent than others under Fenton conditions.[2a,b] We have addressed this question by a combinatorial chemistry approach and present our results here.

To analyse the sequence-dependency of peptide damage under Fenton conditions we used a split-and-mix peptide library where each peptide is flanked by a fluorophor (anthranilic acid, Abz) at the C-terminal side and a quencher (nitrotyrosine, Tyr-(NO_2)) at the N-terminal side (Fig. 1).[3] Beads appear dark as long as the peptides are intact, and fluoresce upon peptide cleavage since the quencher is then no longer covalently attached to the fluorophor.

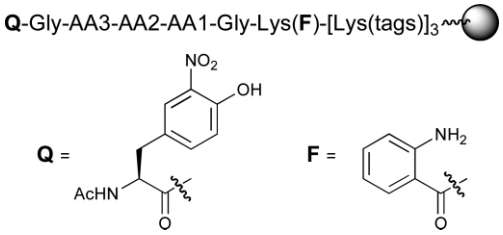

Fig. 1 Fluorophor-quencher flanked peptide library.

The library was prepared on Tentagel resin[4] (PSPEG) by encoded[5] split-and-mix synthesis[6] employing 31 D- and L-amino acids in each position.[7] Hence, the library contained maximally 31^3 = 29791 different peptides. When this library was suspended in a 30 mM aqueous solution of $FeCl_3 \cdot 6H_2O$, washed with water and then treated with H_2O_2 (80 mM) and sodium ascorbate (40 mM) in MES buffer (200 mM) at pH 5 for 15 min, several fluorescent beads (≈ 3 out of 100) were observed (Fig. 2).[8] Isolation of approximately fifty of the most fluorescent beads and analysis of their encoding electrophoric tag molecules by gas chromatography using electron capture detection[5] revealed remarkable selectivities:

Table 1 Peptide cleavage selectivities observed in the screening of the library Ac-Tyr(NO_2)-Gly-AA3-AA2-AA1-Gly-Lys(Abz)-linker-PSPEG under Fenton conditions.[a]

AA3	AA2	AA1	Freq. found[b]
L/D-Glu/Asp	L/D-Glu/Asp	X	34%
X	L/D-Glu/Asp	L/D-Glu/Asp	46%
L/D-Glu/Asp	X	L/D-Glu/Asp	13%

[a] L/D-Glu/Asp = can be either L-Glu, D-Glu, L-Asp or D-Asp, X = random amino acid. [b] The frequency found column lists how often the indicated peptide sequence occurred among the fluorescent beads.

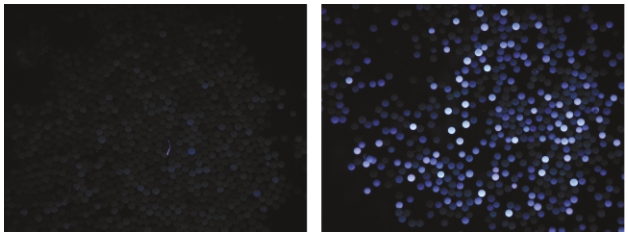

Fig. 2 Fluorophor-quencher flanked peptide library before (left) and after (right) treatment under Fenton conditions.

almost each bead contained two acidic amino acids, either Asp or Glu (Table 1).

This combinatorial experiment demonstrates that acid-rich peptides are cleaved under these Fenton-like conditions to a higher extent than other peptides.[9] To analyze whether the acid-rich peptides also bind to Fe-ions better than other peptides the library was once more incubated with the $FeCl_3 \cdot 6H_2O$ solution, washed and then stained with KSCN.[10] The intensely red $Fe(SCN)_3$ complex was formed on approximately three out of 100 beads. Analysis of the peptides on several of the red beads revealed again sequences with two neighbouring acidic amino acids.[11] These results demonstrate that Fe^{3+}-ions are bound by acid-rich peptides preferentially. This suggests that upon reduction of Fe(III) to Fe(II) by ascorbic acid followed by reaction with H_2O_2 radicals are generated in close vicinity of the Fe^{2+}-ions and therefore damage acid-rich peptides more than others.

To gain further insight into the observed sequence dependent peptide damage we examined the relative cleavage rates of peptides with two glutamic acid residues and negative control peptides that contain glycines in place of the glutamic acids. The studies were performed with the peptides still bound to the solid support and in solution.

For the studies with solid supported peptides, we prepared the peptides Ac-Lys(Abz)-[Gly]$_3$-Glu-Glu-NH$(CH_2)_5$CO-PSPEG **1** and Ac-Lys(Abz)-[Gly]$_3$-Gly-Gly-NH$(CH_2)_5$CO-PSPEG **2**. The N-terminal anthranilic acid was designed to serve as a fluorescence probe that will be released from the solid support into the surrounding solution upon cleavage of the peptide and that can be easily detected by fluorescence spectroscopy. Peptides **1** and **2** were treated under the same Fenton-like conditions as used for the library experiment. After quenching of excess H_2O_2 with MnO_2 and filtration the fluorescence of the filtrates was measured. Remarkably, an intense fluorescence was observed in the filtrate of the glutamic acid containing peptide **1**, while the filtrate of the negative control peptide **2** showed almost no fluorescence (Fig. 3). These results demonstrate that solid supported peptides with two neighbouring glutamic acids are cleaved to a much higher extent (50–100 fold) than peptides with glycines in place of the glutamic acids.

For the studies in solution the fluorophor-marked peptides Ac-Lys(Abz)-[Gly]$_3$-Glu-Glu-NH$(CH_2)_5$CONHPr **3** and Ac-Lys(Abz)-[Gly]$_3$-Gly-Gly-NH$(CH_2)_5$CONHPr **4** were prepared.† To examine their relative damage rates, **3** and **4** were dissolved in MES buffer (200 mM, pH 5) and an equimolar amount of $FeCl_3$ was added followed by solutions of sodium ascorbate (10 equiv.) and H_2O_2 (100 equiv.). After 15 min the reaction mixtures were

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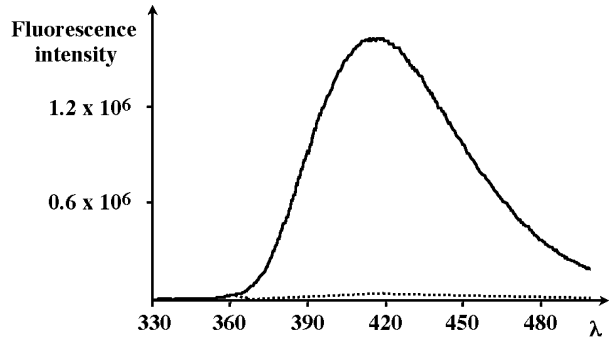

Fig. 3 Fluorescence observed in the filtrates of peptides **1** (solid line) and **2** (dotted line) after reaction under Fenton conditions.

quenched by the addition of MnO_2, filtered and analysed by HPLC with UV or fluorescence detection. The HPLC chromatograms showed a decrease of the peptide signals after the reaction compared to untreated samples. The decrease amounted to approximately 50% for the glutamic acid containing peptide **3** while the glycine containing peptide **4** was damaged to only 5%. Cleavage products were neither observed by HPLC analysis using UV detection nor by HPLC-MS using fluorescence detection.[12] This suggests that the damaged peptides are broken up into fragments that are too small to allow for their analysis by HPLC.

Our results demonstrate that acid-rich peptides are damaged to a greater extent than others under Fenton conditions. Acid-rich peptides also complex Fe-ions better than other tripeptides. The reaction of the complexed Fe^{2+}-ions with H_2O_2 therefore generates radicals in close proximity to the acid-rich peptides. As a result these peptides are damaged more than others under Fenton conditions.

Notes and references

† Peptides **3** and **4** were prepared on Wang resin by the standard Fmoc-protocol, cleaved off the solid support with propylamine in CH_2Cl_2 (1 : 3) and deprotected with a solution of TFA : H_2O : $^iPr_3SiH$ (95 : 2.5 : 2.5). **3**: ^{1}H NMR (500 MHz, CD_3OD, 25 °C): δ = 7.55 (d, J = 7.9 Hz, 1H; Abz), 7.32 (t, J = 7.6 Hz, 1H; Abz), 6.95 (d, J = 7.9 Hz, 1H; Abz), 6.89 (t, J = 6.6 Hz, 1H; Abz), 4.32 (m, 2H; Glu), 4.26 (dd, J = 8.5, 5.8 Hz, 1H; Lys), 3.89 (m, 6H; Gly), 3.36 (t, J = 6.8 Hz, 2H; Lys), 3.17 (m, 2H; Ahx), 3.11 (t, J = 7.2 Hz, 2H; NHC**H**$_2$CH$_2$CH$_3$), 2.40 (m, 4H; Glu), 2.17 (t, J = 7.5 Hz, 2H; Ahx), 2.12 (m, 2H; Glu), 1.98 (m, 2H; Glu), 1.98 (s, 3H; Ac), 1.85 (m, 1H; Lys), 1.72 (m, 1H; Lys), 1.64 (ψq, J = 7.0 Hz, 2H; Lys), 1.60 (ψq, J = 7.6 Hz, 2H; Ahx), 1.48 (m, 6H; NHCH$_2$C**H**$_2$CH$_3$, Ahx, Lys), 1.33 (m, 2H; Ahx), 0.91 (t, J = 7.5 Hz, 3H; NHCH$_2$CH$_2$C**H**$_3$); ^{13}C NMR (500 MHz, CD_3OD, 25 °C): δ = 175.2, 175.1, 174.7, 174.6, 174.1, 172.6, 172.3, 172.0, 171.2, 171.1, 170.9, 146.4, 131.9, 127.8, 126.5, 119.8, 119.7, 54.0, 53.4, 53.0, 42.5, 42.4, 42.4, 40.7, 38.9, 38.8, 35.6, 30.8, 29.9, 29.8, 28.6, 28.5, 26.6, 26.0, 25.3, 22.8, 22.2, 21.0, 10.3; MS (ESI): m/z (%): 891 (100) $[M]^+$. **4**: ^{1}H NMR (500 MHz, D_2O, 25 °C): δ = 7.25 (dd, J = 7.6, 1.3 Hz, 1H; Abz), 7.18 (dt, J = 8.2, 1.5 Hz, 1H; Abz), 6.74 (d, J = 8.1 Hz, 1H; Abz), 6.69 (dt, J = 8.6, 1.2 Hz, 1H; Abz), 4.12 (dd, J = 8.5, 5.8 Hz, 1H; Lys), 3.78 (m, 10H; Gly), 3.22 (t, J = 6.7 Hz, 2H; Ahx), 3.03 (t, J = 7.3 Hz, 2H; Lys), 2.97 (t, J = 6.9 Hz, 2H; NHC**H**$_2$CH$_2$CH$_3$), 2.06 (t, J = 6.3 Hz, 2H; Ahx), 1.86 (s, 3H; Ac), 1.70 (m, 1H; Lys), 1.61 (m, 1H; Lys), 1.50 (m, 2H, Ahx), 1.42 (m, 2H, Ahx), 1.33 (m, 6H; NHCH$_2$C**H**$_2$CH$_3$, Lys), 1.13 (m, 2H; Ahx), 0.72 (t, J = 7.4 Hz, 3H, NHCH$_2$CH$_2$C**H**$_3$); ^{13}C NMR (500 MHz, D_2O, 25 °C): δ = 175.8, 175.4, 175.2, 172. 2, 172.1, 172.0, 171.9, 132.4, 128.0, 118.7, 118.3, 54.0, 46.6, 42.4, 41.0, 39.1, 39.0, 35.6, 30.3, 27.9, 25.3, 25.0, 22.2, 21.7, 21.5, 10.5; MS (ESI): m/z (%): 747 (100) $[M^+$+H].

1 (*a*) M. J. Davies and R. T. Dean, *Radical-Mediated Protein Oxidation*, Oxford University Press, New York, 1998; (*b*) E. R. Stadtman, *Science*, 1992, **257**, 1220–1224.

2 (*a*) H. A. Headlam and M. J. Davies, *Free Radical Biol. Med.*, 2004, **36**, 1175–1184; (*b*) C. L. Hawkins and M. J. Davies, *Biochim. Biophys. Acta*, 2001, **1504**, 197–219; (*c*) E. Cabiscol, J. Tamarit and J. Ros, *Int. Microbiol.*, 2000, **3**, 3–8; (*d*) R. T. Dean, S. Fu, R. Stocker and M. J. Davies, *Biochem. J.*, 1997, **324**, 1–18.

3 M. Meldal, I. Svendsen, K. Breddam and F. Auzanneau, *Proc. Natl. Acad. Sci. USA*, 1994, **91**, 3314–3318.

4 Tentagel was purchased from the Rapp company (Tübingen, Germany).

5 (*a*) M. H. J. Ohlmeyer, R. N. Swanson, L. W. Dillard, J. C. Reader, G. Asouline, R. Kobayashi, M. H. Wigler and W. C. Still, *Proc. Natl. Acad. Sci. USA*, 1993, **90**, 10922–10926; (*b*) H. P. Nestler, P. Bartlett and W. C. Still, *J. Org. Chem.*, 1994, **59**, 4723–4724.

6 (*a*) Á. Furka, F. Sebestyén, M. Asgedom and G. Dibô, *Int. J. Pept. Protein Res.*, 1991, **37**, 487–493; (*b*) K. S. Lam, S. E. Salmon, E. M. Hersh, V. J. Hruby, W. M. Kazmierski and R. J. Knapp, *Nature*, 1991, **354**, 82–84.

7 AA1–AA3 = Gly, D-Ala, L-Ala, D-Val, L-Val, D-Leu, L-Leu, D-Phe, L-Phe, D-Pro, L-Pro, D-Ser, L-Ser, D-Thr, L-Thr, D-Cys, L-Cys, D-Asp, L-Asp, D-Glu, L-Glu, D-Asn, L-Asn, D-Gln, L-Gln, D-His, L-His, D-Lys, L-Lys, D-Arg, L-Arg. The amino acids Ile, Met, Trp and Tyr were not included in the library.

8 Control experiments revealed that all three components (Fe-ions, ascorbate and H_2O_2) are required to observe fluorescent beads. Highest selectivities were obtained when the beads were washed with a solution of EDTA before treatment under Fenton conditions. MES = 2-(4-morpholino)ethanesulfonic acid.

9 Note that only backbone cleavage is examined and not damage of the side chains.

10 M. B. Francis, N. S. Finney and E. N. Jacobsen, *J. Am. Chem. Soc.*, 1996, **118**, 8983–8984.

11 Complexation constants of peptides with two glutamic acids in solution phase could only be estimated in the range of $K_a = 10^4$ due to the precipitation of Fe-hydroxides at pH > 6. Peptides with glycines in place of glutamic acids did not show any measurable binding to Fe-ions under the same conditions.

12 Quenching of the reactions with tBuOH did also not result in products that could be detected by HPLC analysis.