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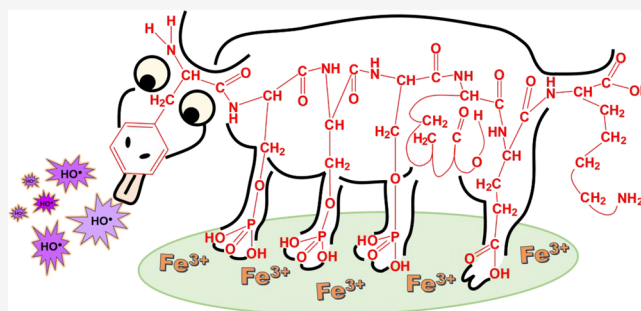


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Supporting Information

ABSTRACT: Antioxidant compounds are chemicals of primary importance, especially for their applications in nutrition and healthcare, thanks to their abilities to prevent oxidation processes and to limit and/or rebalance the oxidative stress, well-known for its impact on a wide variety of diseases. While several biomolecules are well-known for their antioxidant properties (e.g., ascorbic acid, carotenoids, phenolic derivatives), bio-sourced antioxidants have drawn considerable attention in the last decades, especially bioactive peptides, mainly obtained by the hydrolysis process. Antioxidant peptide sequences are mainly identified a posteriori, thanks to fastidious and time-consuming approaches and techniques, limiting the discovery of new efficient peptides. In this context and taking inspiration from nature, we report herein on a new series of three bio-inspired antioxidant peptides derived from the milk protein casein. These phosphopeptides, designed to chelate the redox-active iron(III) and forming highly soluble complexes up to pH 9, act both as *indirect* (i.e., inhibition of the metal redox activity) and *direct* (i.e., radical scavenging) antioxidants.



INTRODUCTION

Reactive oxygen species (or ROS) are essential highly reactive components for the proper functioning of the body, acting as signaling molecules to regulate biological and physiological processes.^{1,2} In healthy organisms, the control of the ROS concentration is ensured by a complex endogenous antioxidant defense system involving enzymes, proteins, or other chemicals.³ However, the presence of endogenous or exogenous stresses can trigger a dysregulation of the ROS homeostasis, inducing the so-called oxidative stress reported to cause a wide variety of diseases.^{4,5} In order to prevent oxidative stress and its detrimental effects, the intake of exogenous antioxidants is an efficient strategy to rebalance the concentration of ROS. Depending on their inherent structure, antioxidants act according to two main modes of action: (i) a *direct mode of action* whereby the antioxidants directly react with the ROS;^{3,6,7} (ii) an *indirect mode of action* whereby the antioxidants hinder the biometal-induced redox aerobic process *via* the chelation of specific transition metals. Indeed, *in vivo*, copper and iron are involved in the production of ROS through the catalytic reduction of molecular oxygen (O_2) to the superoxide anion radical ($O_2^{\cdot-}$), and subsequently to hydrogen peroxide (H_2O_2) and a hydroxyl radical (HO^{\cdot}) (Figure 1a). Thus, their chelation induces a decrease of the ROS production and positive effects on ROS-mediated pathologies.^{3,8–10} Numerous metal-chelating antioxidants have been reported^{3,8–11} and, recently, peptides have emerged as new bio-sourced antioxidants.^{12–14} Peptides have been already considered as therapeutics offering high selectivity and

effectiveness, safety, and tolerability,^{15,16} and biologically relevant peptides can be synthesized using proven, fast, and efficient methods.¹⁷ Up until now, the majority of antioxidant peptides have been identified through tandem LC/MS/MS analyses from protein hydrolysates of natural resources^{12–14,18,19} known for their antioxidant properties. However, this serendipitous approach is hazardous, fastidious, and time-consuming, severely limiting the discovery of new effective antioxidant peptides. Thus, we believe that a more rational approach based on a finely tailored bio-inspired molecular design could achieve the same goal in a more judicious and efficient way. While a large body of work concerning copper-chelating peptides has been reported in the recent years,^{20,21} the development and studies of iron-chelating peptides are still in their infancy and remain challenging. However, iron is present in the body in iron labile pools in which iron is not or slightly chelated and therefore accessible to exogenous antioxidants,^{22,23} including in the lysosomes where iron is in its free Fe(III) form.^{22,24} For these reasons and because Fe(III) is in a biologically unusual +III oxidation state, we focus our attention on the development of antioxidant Fe(III)-

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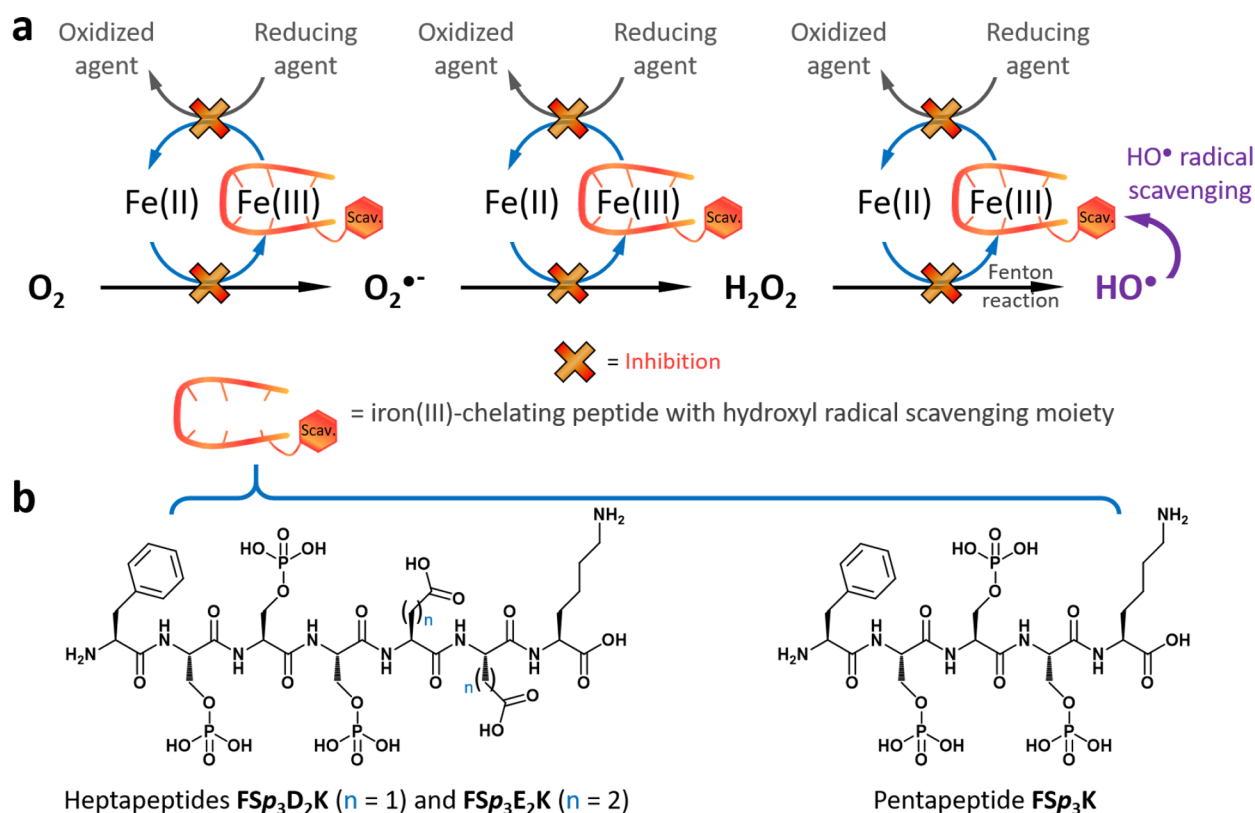


Figure 1. (a) Schematic representation of the *indirect* (i.e., inhibition of the metal redox activity) and *direct* (i.e., radical scavenging) antioxidants mode of actions and (b) chemical structures of the three phosphopeptides studied.

69 chelating peptides with both indirect and direct modes of
70 action (Figure 1a). While a first series of efficient antioxidant
71 pentapeptide has been previously reported by our group,²⁵ we
72 would like to report herein on new bioinspired phosphopep-
73 tides offering both high antioxidant properties and high
74 Fe(III)-chelating capabilities.

75 ■ RESULTS AND DISCUSSION

Bioinspired Design and Synthesis of Antioxidant Phosphopeptides. To design such compounds, we took inspiration from dietary phosphoproteins well-known for their iron-chelating abilities, such as the egg yolk phosvitin²⁶ or the milk caseins.²⁷ In particular, caseins are a family of four proteins termed α_{S1} -, α_{S2} -, β -, and κ -caseins in which phosphorylation occurs at the serine residues and reported for their antioxidant properties.^{28,29} Interestingly, a specific recurring sequence of five amino acids, termed cluster, comprising three phosphoserines (noted *Sp*) followed by two glutamic acids (*E*), *i.e.*, an *SpSpSpEE* cluster, appears once in the β -casein and α_{S1} -casein, and twice in the α_{S2} -casein.^{30–32} Based on these observations and on our previous work,²⁵ we design a first heptapeptide *FSp₃E₂K* (Figure 1b) comprised of the above-mentioned casein-derived cluster on which we grafted (i) a lysine (*K*) at the C-term bringing an additional charge to improve the peptide solubility at neutral pH, and (ii) a phenylalanine (*F*) at the N-term to provide a further antioxidant property to the peptide, *i.e.*, a direct mode of action based on the ability of phenylalanine to entrap hydroxyl radicals (*vide infra*). To evaluate the impact of the two acidic side chains on the chelation and antioxidant properties, two other compounds have been considered: the heptapeptide *FSp₃D₂K* in which the glutamic acids have been replaced by

aspartic acids, and the acidic side chain-lacking pentapeptide FSp₃K in which the two E have been removed. The three phosphopeptides were synthesized, purified, and fully characterized (Supporting Information (SI), part 2).

Evaluation of the Direct and Indirect Antioxidant Properties. To evaluate the antioxidant performance of the three Fe(III)-chelating peptides, we carried out a series of three complementary assays, namely, ascorbate,³³ Amplex Red,³⁴ and 3-CCA (for coumarin-3-carboxylic acid)³⁵ tests to measure the iron redox activity (*i.e.*, the reducibility of Fe(III) to Fe(II)) and the formation of H₂O₂ and HO[•], respectively. To assess the antioxidant power of the three Fe(III)-chelating phosphopeptides, two compounds were selected as references: the positive control is the deferoxamine (DFO), an approved siderophore drug used to treat iron overload and exhibiting antioxidant properties both *in vitro*^{36–38} and *in vivo*,³⁹ whereas the negative control is the ethylenediaminetetraacetic acid (EDTA), well-known for its good chelation abilities but weak antioxidant properties.^{36,37,40}

Thus, employing the ascorbate test, a first series of experiments were carried out with 1 equiv Fe(III) for 1 equiv ligand at physiological pH 7.4. As depicted in [Figure 2a](#), the maximum AscH⁻ consumption is observed for EDTA (negative control) with 64.2 μ M consumed after 10 min. Interestingly, when phosphopeptides were used, this value drastically drops to 15.0 μ M for the casein cluster-derived heptapeptide FSp₃E₂K, to 12.7 μ M for the other heptapeptide FSp₃D₂K, and to 11.0 μ M for the shortest FSp₃K. These data highlight the efficiency of the three peptides to reduce the redox process of the metal ion from 77% (FSp₃E₂K) to 83% (FSp₃K) compared to EDTA, even if the positive control DFO appears slightly more efficient (−96%, 2.5 μ M AscH⁻

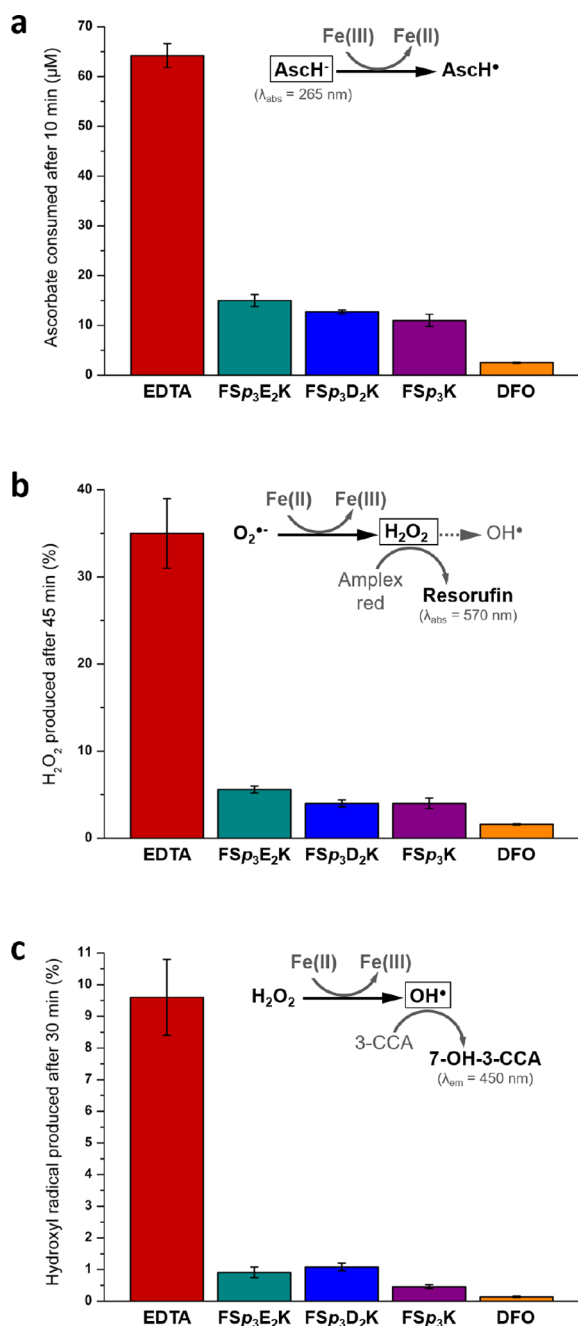


Figure 2. Evaluation of the antioxidant properties of all the phosphopeptides and of the negative (EDTA) and the positive (DFO) reference compounds. The corresponding reactions involved for each assay are inserted in each graph, with their corresponding absorbance or emission wavelengths. All experiments were carried out at pH 7.4 with (a) $[\text{AscH}^-] = [\text{Ligand}] = [\text{Fe(III)}] = 100 \mu\text{M}$, in 50 mM HEPES, (b) $[\text{AscH}^-] = 200 \mu\text{M}$, $[\text{Ligand}] = [\text{Fe(III)}] = 50 \mu\text{M}$ (except $[\text{EDTA}] = [\text{Fe(III)}] = 10 \mu\text{M}$), in 50 mM HEPES, and (c) $[\text{AscH}^-] = 125 \mu\text{M}$, $[\text{Ligand}] = [\text{Fe(III)}] = 50 \mu\text{M}$ (except $[\text{EDTA}] = [\text{Fe(III)}] = 5.0 \mu\text{M}$ and $[\text{AscH}^-] = 12.5 \mu\text{M}$) in water.

contrast, this value drops to 1.6% when the positive control 139 DFO is used. Phosphopeptides also exhibit significant 140 antioxidant properties, with an amount of H₂O₂ produced 141 from 5.6% for FSp₃E₂K to 4.0% for both FSp₃D₂K and FSp₃K. 142 Interestingly, the glutamic acid-incorporating peptide FSp₃E₂K 143 with the closest casein cluster-like sequence has the weaker 144 antioxidant efficiency compared to the two others, as 145 previously observed in the AscH[−] test (Figure 2a). 146 Subsequently, 3-CCA experiments were carried out, and 147 results (Figure 2c) highlight once more that the highest 148 quantity of HO[•] (9.6%, corresponding to the formed HO[•]/ 149 Fe(III)-complex ratio) is formed in the presence of EDTA. 150 Oppositely, DFO and phosphopeptides show better antioxi- 151 dant activities, with a ratio of HO[•] produced of 0.14% for 152 DFO, while this value spans from 1.08 (for FSp₃D₂K) to 0.46% 153 (FSp₃K) for our compounds (Table S3). These experiments 154 confirm that all the three phosphopeptides act as effective 155 antioxidants, slightly less efficient than DFO but highly better 156 than EDTA. 157

However, these peptides have also been designed to behave 158 as direct antioxidants through the entrapment of the most 159 reactive ROS, *i.e.*, the hydroxyl radical HO[•]. Indeed, the 160 oxidation of phenylalanine to tyrosine by HO[•] has already been 161 reported as a marker of oxidative stress.^{41,42} To follow the 162 oxidation of the Phe moiety, a first series of four experiments 163 per peptide was performed by HRMS/MS.²⁵ Thus, the MS 164 spectrum (direct injection) of a stoichiometric peptide/ 165 Fe(III)/AscH[−]/H₂O₂ solution for the heptapeptide FSp₃D₂K 166 (Figure S8, SI for FSp₃E₂K (Figure S14), and FSp₃K (Figure 167 S2)) shows the presence of the native heptapeptide ($m/z =$ 168 513.124 $[\text{M} + 2\text{H}]^{2+}$), the FSp₃D₂K-iron(III) complex ($m/z =$ 169 539.579 $[\text{M-H} + \text{Fe}]^{2+}$), which confirms the Fe(III)-chelating 170 properties of the peptide (discussed hereinafter), and of 171 another peak at $m/z = 521.123$ corresponding to the mono- 172 oxidized peptide $[\text{M} + 2\text{H} + \text{O}]^{2+}$. To separate and precisely 173 analyze each of these species, all the samples were injected in 174 RP-HPLC/HRMS/MS, and the chromatogram for FSp₃D₂K 175 (Figure 3a, SI part 4 for FSp₃E₂K and FSp₃K) reveals the 176 presence of the native peptide (retention time $t_R = 13.4$ min), 177 preceded by a set of three peaks with shorter retention times 178 ($t_R = 10, 11$, and 12.2 min). The latter ones correspond to the 179 mono-oxidized $[\text{M} + 2\text{H} + \text{O}]^{2+}$ species attributable to the 180 oxidation of the phenyl ring of the N-term phenylalanine to its 181 monohydroxy-equivalent as determined by HRMS/MS 182 fragmentation (Figure 3b). The use of HPLC allows us to 183 separate the three phenolic isomers,^{43,44} *i.e.*, the peptides 184 containing the *ortho*-, *meta*-, and *para*-hydroxyphenylalanine, 185 indicative of the reaction of the HO[•] with all positions of the 186 phenyl ring. Interestingly, working with harsher conditions (*i.e.*, 187 FSp₃D₂K/Fe(III)/AscH[−]/H₂O₂ 1/1/10/10) in order to shift 188 the reaction equilibrium, we observed the formation of 189 additional broad peaks (in green, Figure 3c) with shorter t_R 190 $= 7.5$ to 8.9 min characterized by $m/z = 529.116$ 191 corresponding to the di-hydroxylated peptide $[\text{M} + 2\text{H} +$ 192 $2\text{O}]^{2+}$, *i.e.*, the peptide in which the phenylalanine has been 193 oxidized twice (Figure 3d). These experiments confirm the 194 ability of our phosphopeptides to selectively react with HO[•] 195 (control experiments were carried out to discard the possibility 196 of a H₂O₂-induced oxidation, SI part 4) *via* the N-term 197 phenylalanine, validating the relevance of our approach and the 198 direct antioxidant mode of action of our designed peptides. 199 Lastly, the HPLC/HRMS chromatograms also highlight the 200 presence of the mononuclear peptide-Fe(III) complex ($t_R = 201$

consumed, Table S1). To gain further insights into the 133 antioxidant properties of the three phosphopeptides, we then 134 measured their abilities to limit the formation of two distinct 135 ROS, namely, H₂O₂ and HO[•]. Considering the negative 136 control EDTA in the presence of 1 equiv Fe(III), the amount 137 of H₂O₂ formed after 45 min is equal to 35.0% relative to the 138 concentration of the complex (Figure 2b, Table S2). By

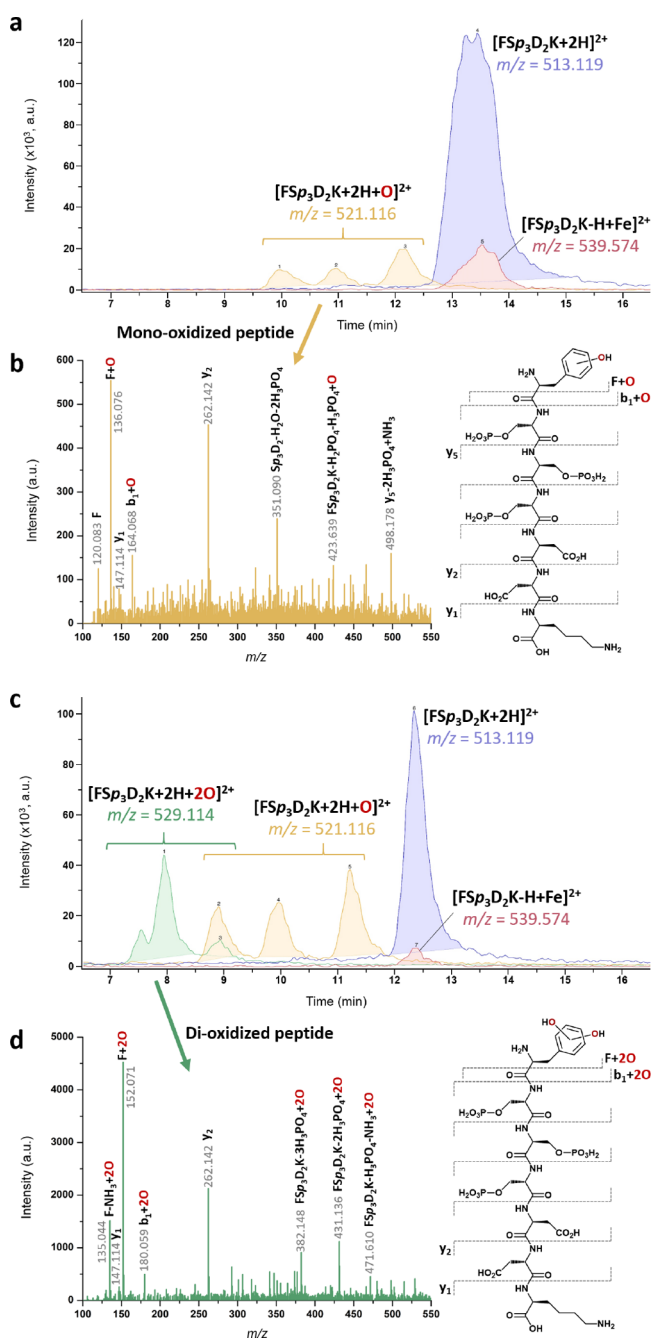


Figure 3. HPLC-HRMS chromatograms highlighting (a) the mono-oxidation of $\text{FSp}_3\text{D}_2\text{K}$ ($[\text{Fe(III)}] = [\text{FSp}_3\text{D}_2\text{K}] = [\text{AspH}^-] = [\text{H}_2\text{O}_2] = 1 \text{ mM}$), and (c) the di-oxidation of $\text{FSp}_3\text{D}_2\text{K}$ ($[\text{Fe(III)}] = [\text{FSp}_3\text{D}_2\text{K}] = 1 \text{ mM}$, $[\text{AspH}^-] = [\text{H}_2\text{O}_2] = 10 \text{ mM}$). Corresponding HRMS/MS spectra of the (b) mono-oxidized peptide at $m/z = 521.116$ and (d) di-oxidized peptide at $m/z = 529.114$. Experiments were carried out at pH 7.4 in water.

pH and to obtain characteristic thermodynamic constants 211 (protonation, complexation) of the peptides and iron(III) 212 complexes. The deprotonation constants of each ligand alone 213 (i.e., in the absence of Fe(III)) are reported in Table 1. 214 t1

Table 1. Overall Protonation Constants ($\log\beta$ H_iL) and Deprotonation Constants (pK) of the Studied Peptides; $T = 298 \text{ K}$, $I = 0.1 \text{ M NaNO}_3$ (Standard Deviations Are in Parentheses)

$\log\beta$	FSp_3K	$\text{FSp}_3\text{E}_2\text{K}$	$\text{FSp}_3\text{D}_2\text{K}$
HL	10.90 (4)	11.36 (1)	11.43 (1)
H_2L	19.16 (6)	19.90 (2)	20.01 (2)
H_3L	26.31 (6)	27.28 (2)	27.43 (2)
H_4L	32.49 (7)	33.86 (2)	34.02 (2)
H_5L	38.18 (8)	39.66 (3)	39.82 (3)
H_6L	42.27 (12)	44.74 (3)	44.72 (3)
H_7L		49.12 (3)	48.85 (3)
H_8L		52.78 (5)	52.37 (5)
pK			
$\text{COOH}_{\text{term}}$	4.09 (12)	3.66 (5)	3.52 (5)
COOH (1)		4.38 (3)	4.13 (3)
COOH (2)		5.08 (3)	4.90 (3)
$\text{PO}_3(\text{OH})^-$ (1)	5.69 (8)	5.80 (3)	5.80 (3)
$\text{PO}_3(\text{OH})^-$ (2)	6.18 (7)	6.58 (2)	6.59 (2)
$\text{PO}_3(\text{OH})^-$ (3)	7.15 (6)	7.38 (2)	7.42 (2)
NH_3^+ (N_{term})	8.26 (6)	8.54 (2)	8.58 (2)
ϵNH_3^+ (Lys)	10.90 (4)	11.36 (1)	11.43 (1)

Depending on the peptide sequence, these ligands have 9 215 (FSp_3K) or 11 ($\text{FSp}_3\text{D}_2\text{K}$, $\text{FSp}_3\text{E}_2\text{K}$) protonable groups, 216 notably one or three carboxylate (COOH/COO^-), three 217 phosphates ($\text{PO}_2(\text{OH})_2/\text{PO}_4^{2-}$), and two amino ($-\text{NH}_3^+/-$ 218 NH_2) groups. The first pK of phosphate groups ($\text{PO}_2(\text{OH})_2/$ 219 $\text{PO}_3(\text{OH})^-$) is too low to be easily determined in titration 220 experiments. Thus, the first measured dissociation step 221 corresponds to the deprotonation of terminal COOH whose 222 value is considerably higher ($\text{pK}_{\text{COOHterm}} = 3.52\text{--}4.09$) than 223 the pK value of free amino acids ($2.0\text{--}2.5$). This increase in 224 the pK values is probably due to the large network of hydrogen 225 bonds formed between the negatively charged phosphate 226 groups (as H acceptors) and the protonated amine and 227 COOH groups (as H donors). This H-bond network has also 228 an impact on the deprotonation process of terminal NH_2 229 ($\text{pK}_{\text{Nterm}} = 8.26\text{--}8.58$) and lysine NH_2 ($\text{pK}_{\text{Lys}} = 10.90\text{--}11.43$) 230 groups, whose pK values are therefore higher than those 231 observed for the first series of pentapeptide FD_3K ($\text{pK}_{\text{Nterm}} =$ 232 7.64 , $\text{pK}_{\text{Lys}} = 10.98$) and FE_3K ($\text{pK}_{\text{Nterm}} = 7.50$, $\text{pK}_{\text{Lys}} =$ 233 10.82).²⁵ In the case of $\text{FSp}_3\text{D}_2\text{K}$, $\text{FSp}_3\text{E}_2\text{K}$ peptides, two 234 additional deprotonation steps were measured corresponding 235 to the dissociation of aspartic and glutamic acid side chains 236 ($\text{pK}_{\text{COOH}(1)} = 4.13$ and 4.38 , $\text{pK}_{\text{COOH}(2)} = 4.90$ and 5.08 for 237 $\text{FSp}_3\text{D}_2\text{K}$ and $\text{FSp}_3\text{E}_2\text{K}$, respectively). These pK values are 238 similar to those measured for the previously studied peptides,²⁵ 239 showing that these groups are not involved in inter- or 240 intramolecular interactions. Regarding the $\text{PO}_3(\text{OH})^-/\text{PO}_4^{2-}$ 241 dissociation processes of three phosphate groups in the peptide 242 sequence, the consecutive pK values are well separated (ΔpK 243 $\approx 0.6\text{--}0.9$) and the first $\text{pK}_{\text{PO}_3(\text{OH})-(1)}$ value ($5.69\text{--}5.80$) 244 corresponds well to those of phosphoserine alone 245 ($\text{pK}_{\text{PO}_3(\text{OH})-\text{serine}} = 5.61$). 246

Then, stability constants ($\log\beta$) and stepwise deprotonation 247 constants (pK) of iron(III) complexes were assessed and are 248 f4

202 13.5 min, $m/z = 539.574$ $[\text{M-H} + \text{Fe}]^{2+}$ for $\text{FSp}_3\text{D}_2\text{K}$ (in red, 203 Figure 3a); see SI part 4 for $\text{FSp}_3\text{E}_2\text{K}$ and FSp_3K , which have 204 been further characterized using potentiometric measurements, 205 circular dichroism (CD), and ^{31}P NMR spectroscopies (SI part 206 5A,B).

207 **Studies on the Phosphopeptides Chelation Proper-** 208 **ties.** First, potentiometric studies were carried out on the 209 synthesized compounds, in the presence and absence of 210 Fe(III) , to both access the species distributions as a function of

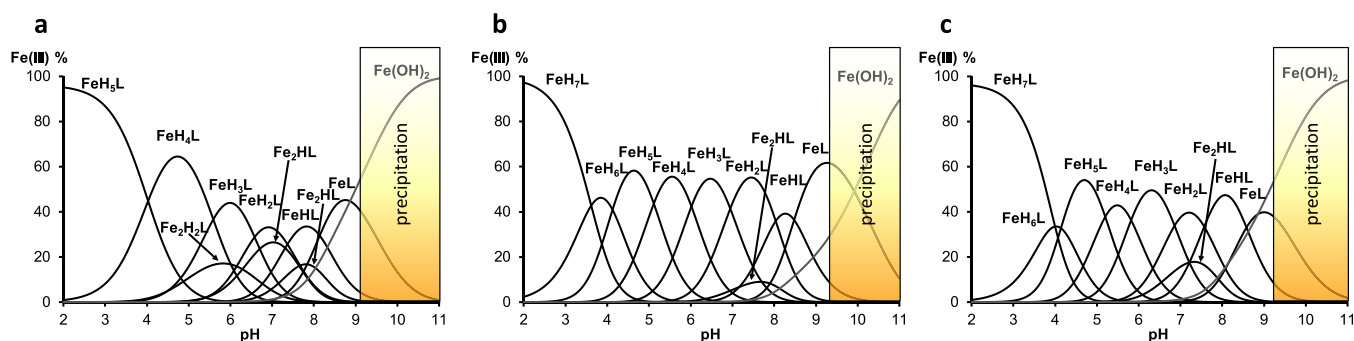


Figure 4. Speciation diagrams of Fe(III)-phosphopeptides 1:1 systems: (a) Fe(III)-FSp₃K, (b) Fe(III)-FSp₃D₂K and (c) Fe(III)-FSp₃E₂K. For all experiments, [peptide]_{tot} = 2.0 mM, *T* = 298 K, and *I* = 0.1 M NaNO₃.

summarized in Table 2 and Figure 4. The results indicate that mono- and binuclear complexes were formed, while bis(ligand) complexes were not observed. For all the three phosphopeptides and at pH 2, the second deprotonation of one phosphate group occurs, meaning that the complexation of iron(III) is already performed by mono- and di-deprotonated phosphate groups. Accordingly, potentiometric titrations start with the species FeH₅L for FSp₃K and FeH₇L for both FSp₃D₂K and FSp₃E₂K. The following steps up to the formation of FeH₄L species correspond to the deprotonation of COOH_{term} for the three phosphopeptides and also to the deprotonations of COOH(1) and COOH(2) in the case of FSp₃D₂K and FSp₃E₂K. The next two dissociation steps belong to the formation of the FeH₃L and FeH₂L complexes where the other two phosphate groups are deprotonated. All these p*K* values from p*K*(FeH₇L/FeH₆L) to p*K*(FeH₃L/FeH₂L) are close to the corresponding p*K* values in the ligands alone (Table 1), demonstrating that the metal ion-promoted dissociation process does not occur. The H-bond network, operating in

the solution of phosphopeptide alone, cannot be formed with terminal NH₃⁺ because the phosphate groups are coordinated to the metal ion; thus, the p*K*_{Nterm} (FeHL formation) value is decreased to 7.37–8.04, a value close to that observed with the previous FD₃K series.²⁵ The last dissociation step corresponds to water deprotonation coordinated to the metal ion with a p*K* value of 8.14, 8.48, and 8.58 for FSp₃K, FSp₃D₂K, and FSp₃E₂K, respectively. At a concentration of 100 μM and at pH 7.4, while small precipitation occurred in the presence of FD₃K, homogeneous solutions were detected with the phosphorylated FSp₃K, FSp₃D₂K, and FSp₃E₂K peptides. At this pH, the p*Fe* value is, for instance, 1.5 logarithmic units higher for FSp₃D₂K (p*Fe* = 14.3) than for FD₃K (p*Fe* = 12.8),²⁵ highlighting that the presence of phosphoserines in the sequences significantly increases the stability of the formed iron(III) complexes and are able to prevent metal ion hydrolysis up to pH 9.2. Based on all these data (potentiometric and spectroscopic, SI part 5), it can be reasonably assumed that the mononuclear 1:1 Fe(III):peptide complexes are formed through the chelation of the metal ion mainly by the phosphate groups, even if other functions (*e.g.* carboxylate) and/or water can be involved in the chelation as previously reported.²⁵ However, to decipher the precise complex structures of these phosphopeptides, further investigations are required. Interestingly, such data both confirm and bring new insights into the role of the casein cluster Sp₃E₂ on the chelation and antioxidant properties of milk casein proteins, as already reported.^{27–32}

Finally, since our phosphopeptides are derived from a specific sequence of casein, a milk protein well-known for its ability to bind calcium mainly through its phosphoserines,^{45,46} we performed competition experiments in the presence of calcium ions. Using the AsCH[−] test, no loss of antioxidant activity is observed upon addition of 1 and 10 equiv of the non-redox active Ca(II) to a 1:1 mixture of Fe:peptide (Table S1); conversely, a sensitive decrease of the ascorbate consumption, *i.e.*, a better antioxidant activity, appears. Additional CD, ³¹P NMR, and ESI-MS studies of the Fe(III):Ca(II):phosphopeptide ternary systems (Figures S23–S27) show that iron remains chelated even with excess calcium and suggest a possible reorganization of the peptides around Fe(III) in the presence of Ca(II) (SI part 5B), confirming the efficiency and selectivity of our bioinspired antioxidant phosphopeptides.

CONCLUSIONS

Thus, we reported herein on the rational design of an original series of bioinspired casein-derived phosphopeptides exhibiting

Table 2. Stability Constants (logβ) and Derived Data (p*K*) for Fe(III) Complexes of the Studied Peptides; *T* = 298 K, *I* = 0.1 M NaNO₃ (Standard Deviations Are in Parentheses)

logβ	FSp ₃ K	FSp ₃ E ₂ K	FSp ₃ D ₂ K
FeH ₇ L		59.24 (5)	60.32 (4)
FeH ₆ L		55.24 (7)	56.70 (4)
FeH ₅ L	48.19 (6)	51.10 (5)	52.57 (4)
FeH ₄ L	44.16 (5)	45.90 (6)	47.45 (3)
FeH ₃ L	38.57 (6)	40.08 (5)	41.43 (3)
FeH ₂ L	31.99 (7)	33.22 (6)	34.48 (3)
FeHL	24.62 (6)	25.65 (3)	26.44 (2)
FeL	16.48(5)	17.07 (5)	17.96 (3)
Fe ₂ H ₆ L		62.05 (7)	62.83 (6)
Fe ₂ H ₅ L		58.78 (7)	59.99 (4)
Fe ₂ H ₄ L	50.71 (8)	54.81 (6)	56.20 (4)
Fe ₂ H ₃ L	47.70 (6)	49.87 (6)	51.54 (4)
Fe ₂ H ₂ L	43.07 (6)	44.33 (5)	45.86 (4)
Fe ₂ HL	36.78 (6)	37.73 (5)	39.09 (4)
Fe ₂ L	29.13 (10)		
p <i>K</i> (FeH ₇ L/FeH ₆ L)		4.00	3.62
p <i>K</i> (FeH ₆ L/FeH ₅ L)		4.14	4.13
p <i>K</i> (FeH ₅ L/FeH ₄ L)	4.03	5.20	5.12
p <i>K</i> (FeH ₄ L/FeH ₃ L)	5.59	5.82	6.02
p <i>K</i> (FeH ₃ L/FeH ₂ L)	6.58	6.86	6.95
p <i>K</i> (FeH ₂ L/FeHL)	7.37	7.57	8.04
p <i>K</i> (FeHL/FeL)	8.14	8.58	8.48

significant antioxidant properties thanks to a dual mode of action. Indeed, these compounds show high Fe(III)-chelating abilities with stability of the complexes formed up to pH 9.2, leading to indirect antioxidant activities. Concomitantly, the direct antioxidant behavior is provided by the phenylalanine moiety and its inherent abilities to act as radical scavenger, especially for the highly reactive hydroxyl radical, as observed by HRMS experiments. Altogether, these two combined antioxidant effects brought by the phosphopeptides reduce, *inter alia*, the metal redox process to 83% and the formation of HO[•] to −95%.

Also, our study highlights that to design effective antioxidant peptides, the incorporation of aromatic amino acid(s) (herein, phenylalanine) is an easy way to induce a direct antioxidant mode of action (*i.e.*, radical scavenging), while the presence of highly chelating amino acids (herein, phosphoserines) are required to assess both an efficient Fe(III) chelation and effective indirect antioxidant properties.

MATERIAL AND METHODS

Peptide Synthesis (General Procedure). The three peptides were synthesized at a 200 μ mol scale using an automated ResPep XL synthesizer (Intavis AG) using an Fmoc/tBu strategy and double couplings for each amino acid. Preloaded Wang-resin (0.70 mmol·g^{−1}, 200–400 mesh) was used. The standard conditions for each coupling were Fmoc-amino acid (5 equivalents), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium tetrafluoroborate (HBTU, 5 equivalents), and 4-methylmorpholine (NMM, 10 equivalents) in dimethylformamide (DMF) and *N*-methyl-2-pyrrolidone (NMP), with a coupling time of 60 min. Fmoc-Ser(PO(OBzl)OH)-OH was introduced at 4.0 equiv using HBTU (4.0 equiv), NMM (8.0 equiv) in DMF, and NMP. Fmoc-deprotection steps were carried out using a 20% piperidine solution in DMF (3 \times 15 min), and final cleavages were achieved using a trifluoroacetic acid/triisopropylsilane/water (92.5/5/2.5) mixture (3 h, 500 rpm). The crude peptides were precipitated from cold diethylether (−20 °C), centrifuged (3 \times 5000 rpm, 5 min) washed with cold diethylether, dried under reduced pressure, resolubilized in water, and finally, lyophilized. Then, peptides were dissolved in a solvent A, sonicated, and purified by semi-preparative HPLC equipped with a Nucleosil (Macherey–Nagel) 100-5 C₁₈ 250 \times 21 mm column using solvent A (95% water, 5% acetonitrile, 0.1% trifluoroacetic acid) and solvent B (100% acetonitrile, 0.1% trifluoroacetic acid) with a UV detection at 214 nm. For FSp₃K, the same procedure was applied except solvent A, which is 97% water, 3% acetonitrile, and 0.1% trifluoroacetic acid. The resulting solutions were evaporated under reduced pressure and were double-lyophilized. The purity of each peptide was evaluated by analytical reversed-phase HPLC equipped with a Nucleosil (Macherey–Nagel) 100-5 C₁₈ 250 \times 4.6 mm column using solvent A' (97% water, 3% acetonitrile, 0.1% formic acid) and solvent B' (100% acetonitrile, 0.1% formic acid) with a UV detection at 214 nm. Peptides were obtained with 31% (FSp₃E₂K), 37% (FSp₃D₂K), and 38% (FSp₃K) yield. All chemical characterizations (NMR, HRMS, and HPLC) are provided in the SI.

Antioxidant Activity Assays (General Procedure). HEPES (50 mM, pH 7.4) buffer was treated on a Chelex100 to remove trace metal contaminants from the solution and prevent unwanted reactions before the different tests. The stock solution at 2 mM of iron(III)-nitrate was prepared from an analytical grade reagent in an acidic media (0.1 M HNO₃) to keep iron(III) ion in solution; its exact concentration was checked spectrophotometrically using complexometric titration. The stock solution at 2 mM of calcium(II)-chloride was prepared in H₂O, and the concentration was checked as the same method as in the case of iron(III). Peptides, EDTA, and DFO ligand stock solutions at 2 mM were prepared using HEPES buffer. To perform the tests, a semi-micro quartz cell with a 1 cm optical pathway was used; the sample holder in the spectrophotometer was

thermostated at 25 °C by a Peltier temperature controller. For all the experiments, the iron(III)-complexes were prepared 20 min before each antioxidant analysis to assure a complete chelation of the metal ion.

Ascorbate Tests. For a standard assay, a 5 mM ascorbate stock solution was freshly prepared using HEPES buffer at pH 7.4. The absorbance of ascorbate was followed over 10 min at 265 nm (ϵ = 14,500 M^{−1} cm^{−1}), and at least three independent measurements were carried out. In a total volume of 1 mL, the final concentration of the components was 100 μ M with 1:1:1 AsCH[−]:ligand:Fe(III) ratio. In the ascorbate test and in the competition study with Ca(II), the final concentration of the components was also 100 μ M with a 1:1:1 (or 2 or 10) AsCH[−]:ligand:Ca(II) ratio or with a 1:1:1:1 (or 10) AsCH[−]:ligand:Fe(III):Ca(II) ratio. The pH was checked at the end of each assay. The reaction was triggered by the addition of ascorbate. As a reference reaction, AsCH[−] oxidation by ligands alone was also studied. The initial ascorbate oxidation rate was calculated from the slope of [AsCH[−]] = $f(t)$ (Figure S1).

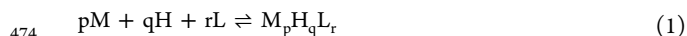
Amplex Red Assays. The H₂O₂ production was detected by an Amplex Red reagent in the presence of horseradish peroxidase (HRP) forming the compound Resorufin with λ_{abs} = 570 nm. For this assay, all components except the iron(III) ion and Amplex Red reagent were dissolved in 50 mM HEPES buffers. The Amplex Red reagent was dissolved in a DMSO:buffer mixture at a 1:4 ratio. A working solution was first prepared containing 0.8 U/mL HRP and 200 μ M Amplex Red reagent in a light-protected tube. A total of 50 μ L of this solution was placed in the total volume of 1 mL of reaction mixture. The final concentration of the AsCH[−] was 200 μ M, and the ligand (peptides or DFO):Fe(III):AsCH[−] ratio was 1:1:4. In the case of EDTA, a ratio of 1:1:20 of EDTA:Fe(III):AsCH[−] was applied. The AsCH[−] was the last reagent added, thus initiating the reaction between O₂ and Fe(III); the pH was checked at the end of each reaction. A negative control containing only the working solution in buffer was necessary in order to monitor the background absorbance of the Amplex Red itself. The Resorufin formation was monitored by visible absorption spectroscopy at 570 nm at RT over 45 min. To determine the H₂O₂ concentration, a standard calibration curve was used. To obtain this calibration curve, a 20 mM hydrogen peroxide solution was previously titrated by permanganometry and then diluted successively. The results are summarized in Table S2.

Coumarin-3-carboxylic Acid (3-CCA) Assay. The HEPES buffers disturbed the fluorescence spectra and also had efficient HO[•] radical scavenging activity, thus pure water treated on Chelex100 was used as solvent and the pH was adjusted by adding 0.1 M NaOH. Thus, all the reagents were dissolved in Milli-Q water except the 3-CCA, since it was only soluble in phosphate buffer (50 mM, pH 7.4). The concentration of the 3-CCA stock solution was 5 mM. In the total volume of 2 mL, the final concentrations of the ascorbate and 3-CCA were both 125 μ M, and the ratio of the reaction mixture was ligand (peptides, EDTA or DFO):Fe(III):AsCH[−] = 1:1:2.5. The pH was checked at the end of the measurement. In the case of EDTA, the amount of all components was 10 times less than that of the other ligand systems (in order to avoid the saturation of the signal due to the high activity of EDTA). For the greater reproducibility of the measured data, each measurement was started by monitoring a blank sample (without any ascorbate) for 5 min, and then the ascorbate was added to the sample. The reaction between 3-CCA and hydroxyl radical generating the fluorescent compound 7-OH-3-CCA was followed over 30 min at 25 °C at λ_{em} = 450 nm (λ_{ex} = 395 nm) by spectrofluorometry. The HO[•] concentration was determined by a standard calibration curve using commercially available 7-OH-3-CCA.

High Resolution Mass Spectroscopy Studies. For direct injections (HRMS, HRMS/MS, and LC-HRMS experiments), the reaction mixtures were prepared at room temperature in a total volume of 1 mL at pH 7.4 in water (pH was adjusted by 0.1 M NaOH) and analyzed after 24 h. Five different mixtures were studied, including peptide alone, peptide + oxidant (H₂O₂), peptide + Fe(III) + oxidant (H₂O₂), peptide + Fe(III) + oxidant (H₂O₂) + ascorbate, and also, as another control without oxidant, peptide + Fe(III) + ascorbate. Similarly to antioxidant assays, the iron(III)-complexes

451 were prepared 20 min before the addition of the redox agent(s)
 452 (H_2O_2 and/or ascorbate), to assure a complete chelation of the metal.
 453 LC-HRMS experiments were carried out using solvent A (95% water,
 454 5% acetonitrile, 0.1% trifluoroacetic acid) and solvent B (100%
 455 acetonitrile, 0.1% trifluoroacetic acid) with a 15 min linear gradient
 456 (0.5% to 8% solvent B) followed by another 10 min linear gradient
 457 (up to 50% B).

458 **Potentiometric Measurements.** The pH-potentiometric titra-
 459 tions were investigated in the pH range of 2.0–11.0 at $I = 0.1 \text{ M}$
 460 NaNO_3 ionic force and at $T = 298.0 \pm 0.1 \text{ K}$. A Dosimat 715
 461 (Metrohm) automatic burette and pH-meter equipped with a semi-
 462 micro combined electrode (Metrohm) was used for the titration. The
 463 initial concentration of peptides was $1.5 \times 10^{-3} \text{ M}$, using 1:1, 1:2, and
 464 2:1 metal-to-ligand ratios. The titrations were performed with a
 465 carbonate free stock solution of sodium hydroxide at known
 466 concentration. During the titration, argon was bubbled through the
 467 samples to ensure the absence of oxygen and carbon dioxide. The
 468 recorded pH readings were converted to hydrogen ion concentration
 469 as described by Irving *et al.*⁴⁷ Protonation constants of the ligands and
 470 the overall stability constants ($\log \beta_{\text{pqr}}$) of the complexes were
 471 calculated from at least three independent titrations (*ca.* 70 data
 472 points per titration) by means of the general computational programs,
 473 PSEQUAD⁴⁸ and SUPERQUAD using eqs 1 and 2.



$$475 \quad \beta_{\text{pqr}} = \frac{[\text{M}_\text{p}\text{H}_\text{q}\text{L}_\text{r}]}{[\text{M}]^\text{p}[\text{H}]^\text{q}[\text{L}]^\text{r}} \quad (2)$$

476 ■ ASSOCIATED CONTENT

477 ■ Supporting Information

478 The Supporting Information is available free of charge at
 479 <https://pubs.acs.org/doi/10.1021/acs.inorgchem.1c03085>.

480 Chemical analysis of synthesized peptides, results of
 481 antioxidant activity assays, high-resolution mass spec-
 482 troscopy studies, and complexation studies (PDF)

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