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Milk-derived bioactive peptides exhibit antioxidant activity through the Keap1-Nrf2 signaling pathway

Federica Tonolo[a], Alessandra Folda[a], Luca Cesaro[a], Valeria Scalcon[a], Oriano Marin[a], Stefania Ferro[a], Alberto Bindoli[b], Maria Pia Rigobello[a,*]

[a] University of Padova, Department of Biomedical Sciences, via Ugo Bassi 58/b, 35131 Padova, Italy
[b] Institute of Neuroscience, CNR, viale G. Colombo 3, 35131 Padova, Italy

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ABSTRACT

Bioactive peptides are relevant nutritional factors that exhibit many functions including antioxidant, anti-hypertensive, anticancer and antimicrobial properties. In this paper, four synthetic peptides ARHPHPHLSFM (A-11-M), AVPYPQR (A-7-R), NPYVPR (N-6-R) and KVLPVPEK (K-8-K) with sequences present in milk proteins were examined for their antioxidant properties. The compounds show moderate free radical scavenging activity in the ABTS and crocin assays (A-7-R and N-6-R) and lipid peroxidation inhibition in Caco-2 cells (N-6-R and K-8-K). All peptides, in particular K-8-K, activate the Keap1-Nrf2 system by allowing the translocation of the transcription factor Nrf2 from the cytosol to nucleus. This activation triggers the overexpression of the antioxidant enzymes Trx1, TrxR1, GR, NQO1 and SOD1. Furthermore, molecular modeling shows that K-8-K is able to hinder the interaction of Nrf2 with Keap1. The reported results show that the antioxidant action in cells of these bioactive peptides is mostly due to the activation of Keap1-Nrf2 signaling pathway.

1. Introduction

Milk is a source of several molecules including bioactive peptides (Dziuba & Dziuba, 2014; Korhonen, 2009; Nongonierma & FitzGerald, 2015; Pihlanto, 2006) generated by proteolysis of casein (α-, β-, γ- and κ-casein) and whey proteins (β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulins, lactoferrin and protease-peptone fractions). These peptides are inactive in the native form of the parent milk proteins and can exert their effects only after hydrolysis. Among other functions, milk-derived bioactive peptides can act, as anti-hypertensive, anticancer, anti-microbial and antioxidant factors (Egger & Ménard, 2017; Sanchez & Vazquez, 2017). In this work, four antioxidant bioactive peptides are analyzed in order to unravel their role in the regulation of cell redox signalling. Peptides ß-CN f(177-183) with the sequence AVPYPQR (A-7-R) (Rival, Fornaroli, Boeriu, & Wichers, 2001), ß-CN f(169-176)-KVLPVPEK (K-8-K) (Rival et al., 2001), k-CN f (96-106)-ARHPHPHLSFM (A-11-M) (Korhonen & Pihlanto, 2007; Kudoh, Matsuda, Igoshi, & Oki, 2001), corresponding to sequences present in bovine ß-casein and k-casein in addition to peptide k-CN f (53-58) with the sequence NPYVPR (N-6-R) (Tsopmo et al., 2011; Wada & Lönnerdal, 2014), corresponding to a sequence found in human k-casein, were characterized and shown to exhibit antioxidant properties *in vitro*.

In the previous research (Tonolo et al., 2018) these four peptides were shown to be able to decrease reactive oxygen species (ROS) production and protect Caco-2 cells from induced oxidative stress. In addition, in oxidizing conditions, they protect thiol-dependent antioxidant enzymes such as thioredoxin and glutathione reductases. Usually, the antioxidant action of the bioactive peptides is based on their free radical scavenging capability in association with the property of acting as strong chelators of transition metals (Power, Jakeman, & Fitzgerald, 2013). However, it has been recently suggested that the antioxidant action can be also defined as the capability of a specific molecule to increase the amount of the antioxidant enzymes in a process mediated by the Keap1-Nrf2 system (Baird & Dinkova-Kostova, 2011; Forman, Davies, & Ursini, 2014). It is well known that nuclear factor erythroid 2–related factor 2 (Nrf2) migrates to the nucleus where, after binding to the antioxidant response element (ARE), activates a large number of

Abbreviations: ABAP, 2,2′-azobis(2-methylpropionamidine); ABTS, 2, 2′-azinobis(3-ethylbenzothiazoline 6-sulfonate); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, gluthatione reductase; Keap1, Kelch-like ECH-associated protein 1; MDA, malondialdehyde; Nrf2, nuclear factor erythroid 2-related factor 2; NQO1, NAD(P)H quinone dehydrogenase 1; PCNA, proliferating cell nuclear antigen; ROS, reactive oxygen species; TbOOH, *tert*-butyl hydroperoxide; TEAC, Trolox equivalent antioxidant concentration; Trx1, thioredoxin 1; TrxR1, thioredoxin reductase; WB, Western blot

* Corresponding author.
E-mail address: mariapia.rigobello@unipd.it (M.P. Rigobello).

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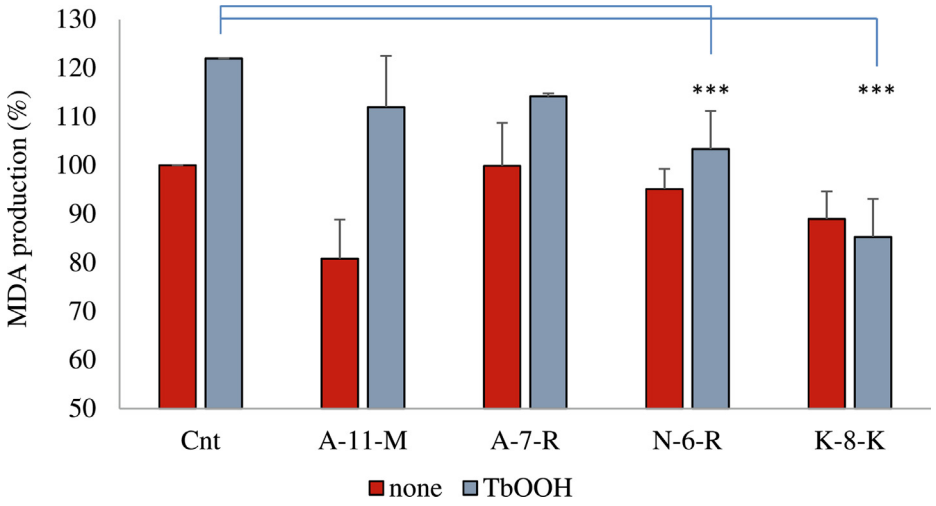

Fig. 1. Estimation of lipid peroxidation as MDA production, in Caco-2 cells pre-treated with A-11-M, A-7-R, N-6-R and K-8-K in the absence or presence of TbOOH. Caco-2 cells (4.5×10^5) were pre-treated with 0.05 mg/mL peptides and MDA formation was estimated fluorometrically as described in Materials and methods, in basal conditions (none, red) and after two hours treatment in the presence of 250 μM TbOOH (gray). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

genes expressing antioxidant enzymes (Baird & Dinkova-Kostova, 2011; Dinkova-Kostova, Kostov, & Canning, 2017). The Kelch-like ECH-associated protein 1 (Keap1) promotes the prompt degradation, via the proteasome system, of the transcription factor Nrf2 by binding to it. Stress conditions, elicited both by oxidant molecules and by electrophilic compounds, are able to induce the activation of Nrf2 due to its dissociation from Keap1. More recently, a novel way of activating Nrf2 was shown to depend on the disruption of the Keap1-Nrf2 protein-protein interaction by the action of specific peptides (Hancock et al., 2012; Lu et al., 2018). For example, a cyclic peptide interacting with Keap1 was reported by Lu et al. (2018) and shown to possess anti-inflammatory properties. In the present paper, we have investigated, in addition to their previously described antioxidant properties (Tonolo et al., 2018), the molecular mechanism of action of the four synthesized peptides, A-11-M, A-7-R, N-6-R and K-8-K, corresponding to peptides available from milk proteins. We report that these peptides, and in particular K-8-K, induce the overexpression of antioxidant enzymes in cultured cells via activation of the Keap1-Nrf2 pathway. This observation is further reinforced by a molecular docking approach.

2. Material and methods

2.1. Materials

Trolox C, 2, 2′-azinobis(3-ethylbenzothiazoline 6-sulfonate) (ABTS), saffron, IGEPAL CA-630, butylated hydroxytoluene (BHT), hemin, cumene hydroperoxide, *n*-butanol, phosphotungstic acid, thiobarbituric acid and potassium persulfate were purchased from Merck-Fluka-Sigma-Aldrich (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were obtained from Gibco (Thermo Fisher Scientific, Massachusetts, USA). Primary mouse monoclonal antibodies for Nrf2 (A10), TrxR1 (B2), Trx1 (A5), SOD1 (24), GR (C10), NQO1 (A180), GAPDH (6C5), PCNA (H2) and goat anti-mouse secondary antibody were purchased from Santa Cruz Biotechnology Inc. (California, USA).

2.2. Peptide synthesis

The peptides A-11-M (ARHPHPHLSFM), A-7-R (AVPYPQR), N-6-R (NPYVPR) and K-8-K (KVLPVPEK) were synthesized by a solid-phase technique as described previously (Tonolo et al., 2018).

2.3. Analysis of antioxidant activity of the four peptides with the ABTS assay

ABTS assay was employed to determine the antioxidant capacity of the four peptides (Perna, Intaglietta, Simonetti, & Gambacorta, 2013; Tonolo et al., 2019). $ABTS^{\bullet+}$ was generated by reaction of 7 mM ABTS with 2.46 mM potassium persulfate and the resulting mixture was maintained at room temperature (RT), in the dark, for 18 h before use. Peptide solutions (0.05 and 0.1 mg/mL, final concentrations) were diluted in water to reach 0.1 mL of final volume and then were treated with 0.1 mL of 0.08 mM $ABTS^{\bullet+}$. The decrease in absorbance was measured at 415 nm with a Tecan plate reader (Tecan Infinite® M200 PRO, Männedorf, CH). A calibration curve was performed with Trolox C and the results are expressed as Trolox C equivalent antioxidant capacity (TEAC).

2.4. Crocin bleaching test

In order to evaluate the peroxyl radical scavenging capacity of the peptides, crocin-bleaching test was carried out. Crocin is able to intercept the peroxyl radicals produced by thermal decomposition of ABAP and bleaches at decreasing rates according to the efficiency of the antioxidant bioactive peptides. For this purpose, alcoholic crocin solution was prepared from saffron following the procedure of Friend and Mayer (1960) and its concentration was estimated using an $\varepsilon_M = 1.33 \times 10^5\ M^{-1}\ s^{-1}$ at 440 nm. The crocin bleaching test was carried out at 40 °C in 0.2 M phosphate buffer (pH 7.0) containing 5% ethanol, in the presence of 12 μM crocin and 5 mM 2,2′-azobis(2-methylpropionamidine) (ABAP) (Bors, Michel, & Saran, 1984; Tubaro, Micossi, & Ursini, 1996). The antioxidant bioactive peptides were added at increasing concentrations starting from 6 μM. The rate of crocin bleaching was followed spectrophotometrically as decrease of absorbance at 440 nm. Bleaching rates were plotted according to the equation $v_0/v = 1 + k_a[A]/k_c[C]$, where v_0 and v are the bleaching rate of crocin in the absence and in the presence of the antioxidant, respectively. [A] is the concentration of the antioxidant and [C] is the concentration of crocin. k_a and k_c are the rate constants for the reaction of the peroxyl radical with the antioxidant and the crocin, respectively. The plot results in a straight line with y intercept equal to one, while the slope corresponds to the ratio of the rate constants k_a/k_c indicating the relative capacity of different antioxidant bioactive peptides to interact with the peroxyl radical.

2.5. Caco-2 cell culture

Caco-2 cells were cultured in DMEM supplemented with 10% FBS, 10,000 units/mL of penicillin and 1 mg/mL of streptomycin at 37 °C in humidified atmosphere containing 5% CO_2. The cells used in this study were between 35 and 60 passages.

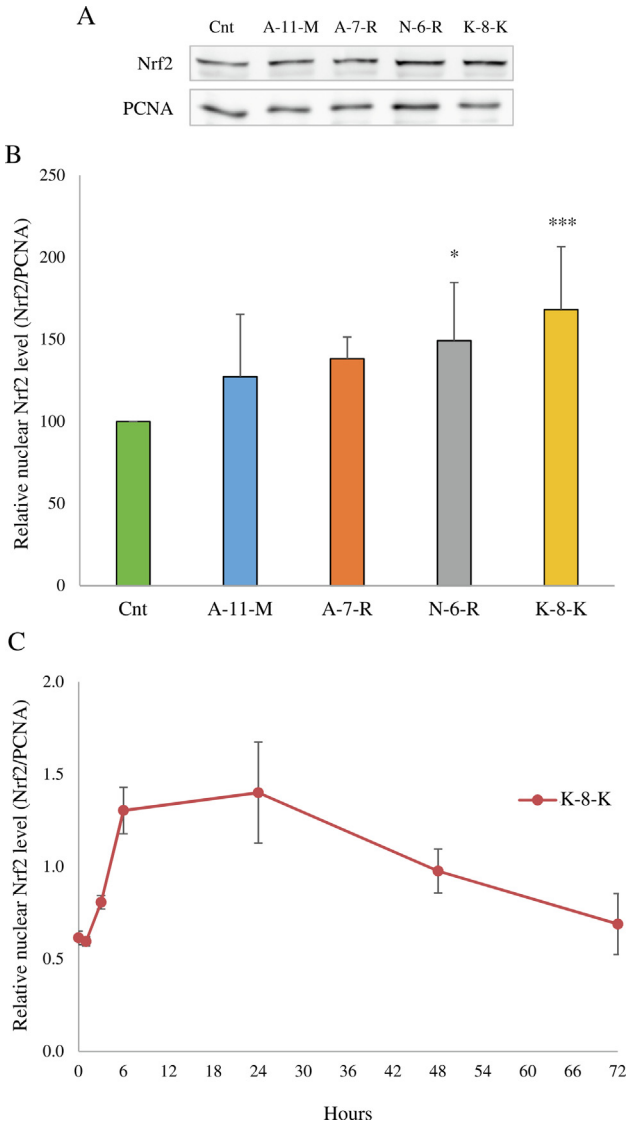

Fig. 2. Nrf2 pathway activation in Caco-2 cells treated with A-11-M, A-7-R, N-6-R and K-8-K (A, B); time scale of Nrf2 nuclear translocation in the presence of K-8-K (C). **A**: Caco-2 cells were treated with 0.05 mg/mL peptides for 24 h. Nuclear fractions were isolated and protein immunoblotted as indicated in Materials and methods. **B**: Densitometric analysis of the above reported WB, using PCNA as loading control. **C**: Cells were treated with K-8-K and the translocation of Nrf2 from the cytosol to the nucleus was estimated after 0, 1, 3, 6, 24, 48 and 72 h.

2.6. Lipid peroxidation assay

The protocol to estimate the lipid peroxidation was referred to Yagi (1984) with some modifications (Tonolo et al., 2019). Briefly, cells were seeded (4.5×10^5) in a 6 well plate and grown for 48 h, pre-treated for 2 h with 0.05 mg/mL of each peptide and subsequently incubated with 250 μM tert-butyl hydroperoxide (TbOOH). After 24 h, cells were harvested, washed with 1 mL of PBS 1X and then disrupted with 1 mL of 0.1 N H_2SO_4 and 150 μL of 10% phosphotungstic acid (PTA) for 10 min at RT. Afterwards, samples were centrifuged at 15,600*g* for 10 min, the supernatants were removed and the pellets were suspended in 1 mL of 0.1 N H_2SO_4 and 150 μL of 10% PTA. After 5 min at RT, the samples were centrifuged as reported above. Supernatants were discarded, the dry pellets dissolved in 0.35% IGEPAL, 0.014% BHT and 0.23% thiobarbituric acid (H_2O/acetic acid, 1:1) (final volume 0.25 mL) and incubated at 95 °C for 60 min. Then, samples were cooled in ice and centrifuged at 15,600*g* for 10 min. The supernatants were treated with 400 μL of n-butanol, vigorously mixed and centrifuged at 15,600*g* for 15 min. The fluorescence of the upper phase, containing the malondialdehyde (MDA) adduct, was analysed fluorometrically at 530 nm (Ex) and 590 nm (Em) using a Tecan plate reader.

2.7. Evaluation of Nrf2 translocation to the nucleus compartment. Isolation of subcellular fractions and WB analysis

To evaluate the translocation of Nrf2 to the nucleus, nuclear and cytosolic fractions were separated by following the procedure described by Yao et al. (2014) with some modifications. Cells (1×10^6) were seeded in T25 flasks and grown for 48 h. Then, cells were pre-treated for 2 h with 0.05 mg/mL of each peptide and subsequently incubated in the presence of 1.5 mM H_2O_2. After 24 h, cells were harvested, washed with 1 mL of PBS 1X and lysed with 100 μL of buffer containing 10 mM Hepes/Tris pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM PMSF, 1 mM NaF and a protease inhibitor cocktail (Complete, Roche®) for 15 min in ice. Afterwards, 20 μL of IGEPAL (5% final concentration) were added to the samples and vigorously mixed for 15 s. Afterward, cell lysates were centrifuged at 1,000*g* for 10 min at 4 °C. The pellet constitutes the nuclear fraction that was dissolved in 20 mM Hepes/Tris (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, in the presence of 0.1 mM PMSF, 1 mM NaF and a protease inhibitor cocktail (Complete, Roche®). Samples were kept on ice for 15 min and vigorously mixed every 2 min for 10–15 s. To further purify nuclear fractions, samples were centrifuged at 20,000*g* for 10 min at 4 °C and the debris was discarded. Proteins were estimated according to Lowry, Rosebrough, Farr, and Randall (1951). The expression level of Nrf2 (nuclear fraction), was determined by Western blot (WB). 30 μg of proteins were subjected to SDS-PAGE (10%). Samples were transferred onto a nitrocellulose membrane (Santa Cruz Biotechnology Inc., California, USA) using Trans Blot® Turbo™ (BIORAD, California, USA) and probed with the primary monoclonal antibody. The WB detection was obtained using ECL system with UVITEC (Alliance Q9 Advanced) equipment. Densitometric analysis of WB was performed using NineAlliance software.

2.8. Analysis of phase II enzymes in Caco-2 cells by WB

Cells treated as reported in paragraph 2.7 were harvested, washed with 1 mL of PBS 1X and then lysed with 150 μL of lysis buffer (RIPA buffer modified), containing 150 mM NaCl, 1% Triton X 100, 0.1% SDS, 0.5% DOC, 1 mM NaF, 1 mM EDTA, 5 mM Tris/HCl (pH 7.4), 0.1 mM PMSF and protease inhibitor cocktail. Then, samples were incubated at 4 °C for 45 min and, after aspiration with a 22-gauge needle, cells lysates were centrifuged for 5 min at 11,600*g* to discard the debris. The amount of protein was determined by Lowry method. The expression levels of superoxide dismutase (SOD1), thioredoxin reductase (TrxR1), thioredoxin 1 (Trx1), glutathione reductase (GR) and NAD(P)H quinone dehydrogenase 1 (NQO1) were determined by immunoblot. Cell lysates (30 μg of proteins) were subjected to SDS-PAGE (12%) and then blotted onto a nitrocellulose membrane and probed with the selected primary antibodies. The WB detection was performed using ECL system with UVITEC (Alliance Q9 Advanced) equipment. Densitometric quantification of phase II enzymes WB was performed using NineAlliance software.

2.9. Evaluation of NQO1 and Trx1 activities in cell lysates

NQO1 and Trx1 are cytosolic antioxidant enzymes strictly related with the Keap1-Nrf2 pathway. Enzymatic activities were measured in Caco-2 cell lysates obtained as above described. For Trx1 determination, 24 μg of proteins were incubated for 40 min at 37 °C in the presence of 300 μM NADPH, 0.2 mg/mL insulin and 31.6 nM thioredoxin

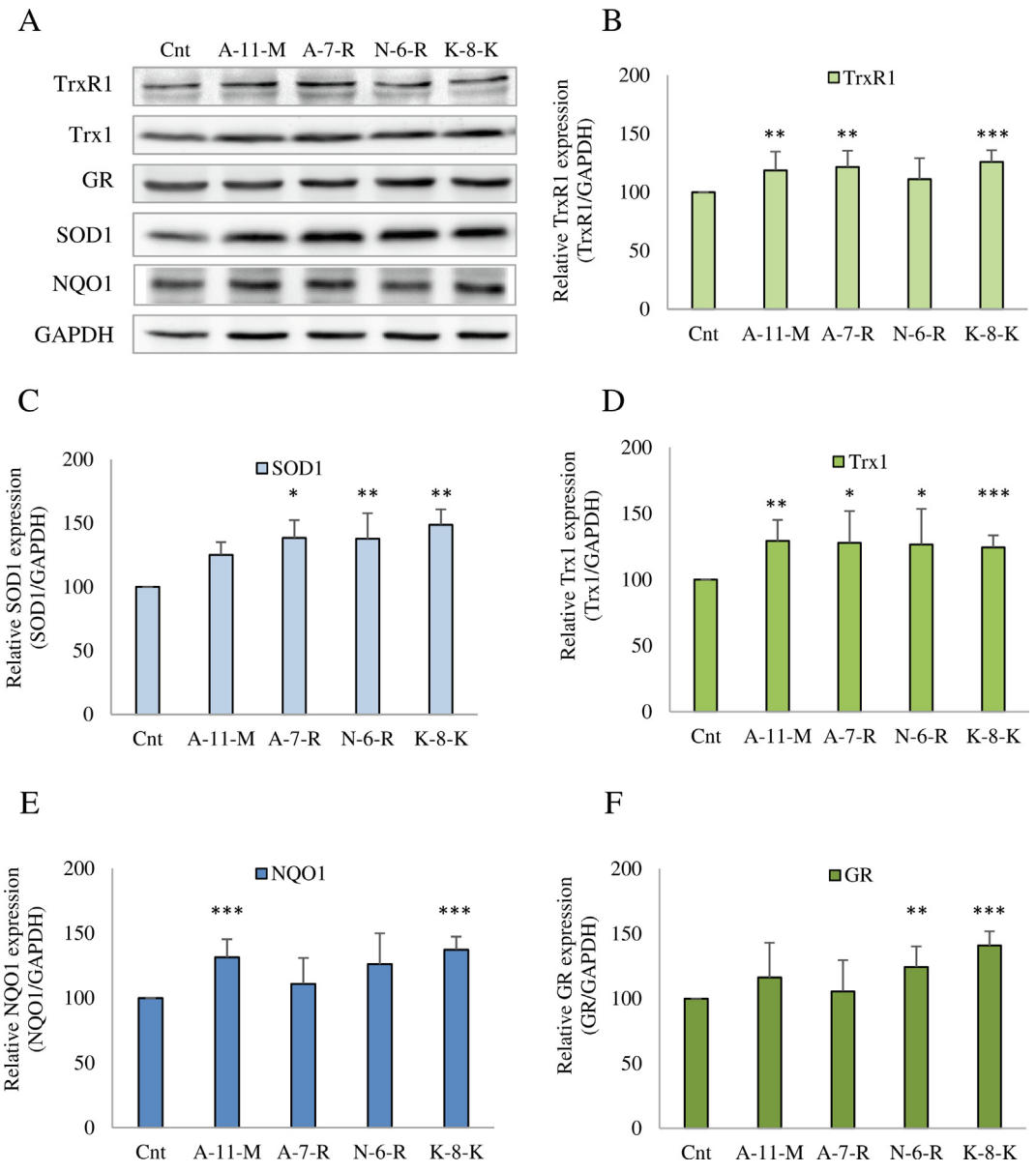

Fig. 3. Overexpression of phase II antioxidant enzymes TrxR1, Trx1, GR, SOD1 and NQO1 in the presence of A-11-M, A-7-R, N-6-R and K-8-K. **A**: Western blot estimation of the Nrf2 downstream enzymes TrxR1, Trx1, GR, SOD1 and NQO1 in Caco-2 cells after incubation with 0.05 mg/mL peptides for 24 h. **B-F**: Densitometric analysis of the above reported WB, using GAPDH as loading control.

reductase in a buffer containing 100 mM Hepes, 15 mM EDTA (pH 7.6) to a final volume of 50 µL. At the end, 200 µL of 0.2 M Tris/HCl (pH 8.1), 1 mM EDTA, 7.2 mM guanidine and 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were added to the samples and absorbance was recorded at 412 nm (Arnér & Holmgren, 2001) using a Tecan Plate reader. NQO1 activity was measured following the decrease of absorbance of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm (Yao et al., 2014). Briefly, cell lysates (30 µg) were incubated in the presence of 200 µM NADPH, 16 µM dicumarol and 20 mM Tris/HCl (pH 7.4) (final volume 250 µL). The reaction was started by adding 40 µM DCPIP and the decrease of absorbance at 600 nm was recorded.

2.10. Molecular docking

The interaction between Keap1 and the most effective peptide, K-8-K, has been investigated using docking analysis. Crystal structure of Kelch domain of Keap1 bound to Neh2 domain of Nrf2 (2FLU) was selected from RCSB PDB database (http://www.rcbs.org) (Berman et al., 2000). Protein structures were prepared for the simulation with HTMD software (Doerr, Harvey, Noé, & De Fabritiis, 2016) and molecular modelling was performed using ACEMD software (Harvey, Giupponi, & De Fabritiis, 2009). Molecular graphics were prepared using Pymol (Delano Scientific LLC, San Carlos, CA, USA) (Baker, Sept, Joseph, Holst, & McCammon, 2001).

2.11. Statistical analysis

The reported values are the mean ± SD of at least three independent experiments. The analysis of variance was performed by multiple comparison test with Tukey-Kramer method (GraphPad InStat 3) and the differences with $P < 0.05$ were considered significant.

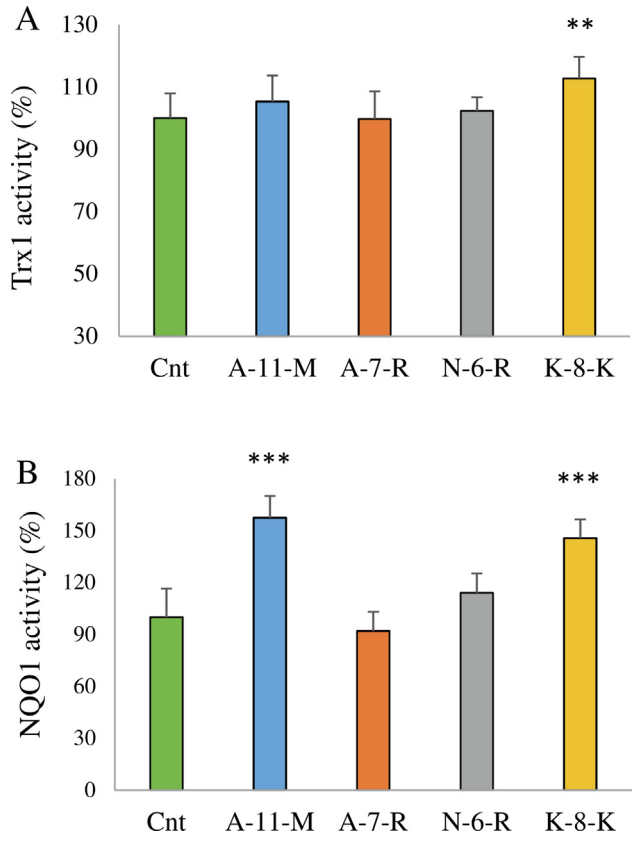

Fig. 4. Trx1 (A) and NQO1 (B) activities in Caco-2 cells treated with the four peptides. The activity of the two antioxidant enzymes was estimated in cell lysates of Caco-2 cells treated with the four peptides (0.05 mg/mL) for 24 h.

3. Results

3.1. The four peptides show moderate antioxidant activity by the ABTS and crocin bleaching assay

The free radical scavenger capability *in vitro* of the four peptides has been evaluated with the ABTS and crocin assays. The first test relies upon the reaction of a relatively stable free radical, such as the cation radical $ABTS^{\cdot+}$, with molecules potentially acting as free radical scavengers. Trolox C, a water soluble form of vitamin E, is taken as reference antioxidant molecule. As presented in Table S1, peptides N-6-R and A-7-R showed some reactivity with the ABTS radical, while K-8-K and A-11-M were ineffective.

Similar results were obtained with the crocin bleaching test (Fig. S1). A flux of hydroperoxyl radicals was produced upon thermal decomposition of the diazocompound ABAP, allowing the estimation of the reaction rate between peroxyl radicals and the various antioxidant molecules. Therefore, the rate constant ratio of crocin with respect to the antioxidant under examination (k_a/k_c) could be measured. All the four peptides were evaluated, but they did not display any competition in the bleaching test with crocin (Fig. S1).

3.2. Lipid peroxidation decreases in Caco-2 cells treated with the four peptides

Then, the potential antioxidant effect of the four peptides was examined by estimating MDA formation in cultured cells treated with TbOOH, which is able to induce lipid peroxidation. MDA is a product of the lipid peroxidation process, as it derives from the fragmentation of unsaturated fatty acids when they are subjected to a free radical attack. Cells were pre-treated for 2 h with the various peptides, and lipid peroxidation was estimated after 24 h in the presence of 250 μM TbOOH as a source of alkoxyl and peroxyl radicals. In Fig. 1 it is apparent that the four peptides did not increase lipid peroxidation in basal conditions but, on the contrary, slightly decreased MDA formation especially with A-11-M. Notably, when lipid peroxidation was induced by the presence of TbOOH, a protective effect was observed, mainly for peptides N-6-R and K-8-K. In particular, N-6-R led to values similar to those of control without TbOOH, or even lower in the case of K-8-K. Indeed, the protection against lipid peroxidation exerted by K-8-K was more than 30% compared to the control in the presence of TbOOH alone.

3.3. Peptides affect translocation of Nrf2 to the nucleus in Caco-2 cells

To elucidate the molecular mechanism by which peptides are able to protect Caco-2 cells against oxidative stress injury, the expression of Nrf2 was evaluated by Western blot analysis. In particular, the involvement of the Keap1-Nrf2 pathway was explored by estimating the translocation to the nucleus of the transcription factor Nrf2. As reported in Fig. 2, all four peptides were able to stimulate the translocation of Nrf2 to the nucleus in Caco-2 cells, with peptide K-8-K being the most active, as shown by densitometric analysis (Fig. 2B). For this estimation, proliferating cell nuclear antigen (PCNA) was employed as loading control. Based on these results, the presence of Nrf2 in the nucleus of cells treated with K-8-K at increasing times was estimated. As shown in Fig. 2C, K-8-K determined a relatively rapid increase of the transcription factor nuclear level at 6 h, reaching its maximum at 24 h of treatment, followed by a progressive decrease.

3.4. The bioactive peptides lead to the overexpression of phase II enzymes

The master transcription factor Nrf2 acts on ARE (antioxidant response element) and activates a large number of genes coding for antioxidant enzymes involved in cell protection against oxidative stress. As indicated in Fig. 3 A, all peptides at 24 h of treatment stimulated the expression of antioxidant genes leading to the downstream biosynthesis of cytosolic superoxide dismutase (SOD1), thioredoxin (Trx1), thioredoxin reductase (TrxR1), glutathione reductase (GR) and NAD(P)H quinone dehydrogenase 1 (NQO1). Densitometric analyses are reported for each enzyme, normalized with GADPH as loading. All these results, once again, indicated that these peptides were able to activate Keap1-Nrf2 pathway leading to the overexpression of the phase II enzymes. In particular, K-8-K exhibited the highest score of significance, denoting its major effect in increasing antioxidant enzymes such as SOD1, GR, Trx1 and NQO1.

3.5. NQO1 and Trx1 activities in Caco-2 cells

To further assess the overexpression of the phase II enzymes, NQO1 and Trx1 activities were estimated. A significant increase of both Trx1 and NQO1 enzymatic activities was detected in Caco-2 cells treated with K-8-K for 24 h (Fig. 4A and 4B, respectively). In addition, A-11-M was able to induce an increase of NQO1 activity, in agreement with the overexpression of the enzyme shown above (Fig. 3E).

3.6. Molecular modelling of the interaction between Keap1 and K-8-K

Docking analysis was performed in order to investigate the interaction of Keap1-Nrf2 complex with the most effective peptide, K-8-K. First, when comparing the structure of Keap1-Nrf2 complex (Fig. 5A) with that of Keap1-K-8-K (Fig. 5B), after 80 ns of simulation it was evident that the peptide interacted with Keap1 in the same motif between Neh domain of Nrf2 and Keap1. As shown in Fig. 5D, the glutamic acid (Glu) of the peptide is stabilized by a hydrogen bond with the arginine 380 (Arg 380) of Keap1. Moreover, the carbonylic groups of the peptide backbone formed hydrogen bonds with other amino acids

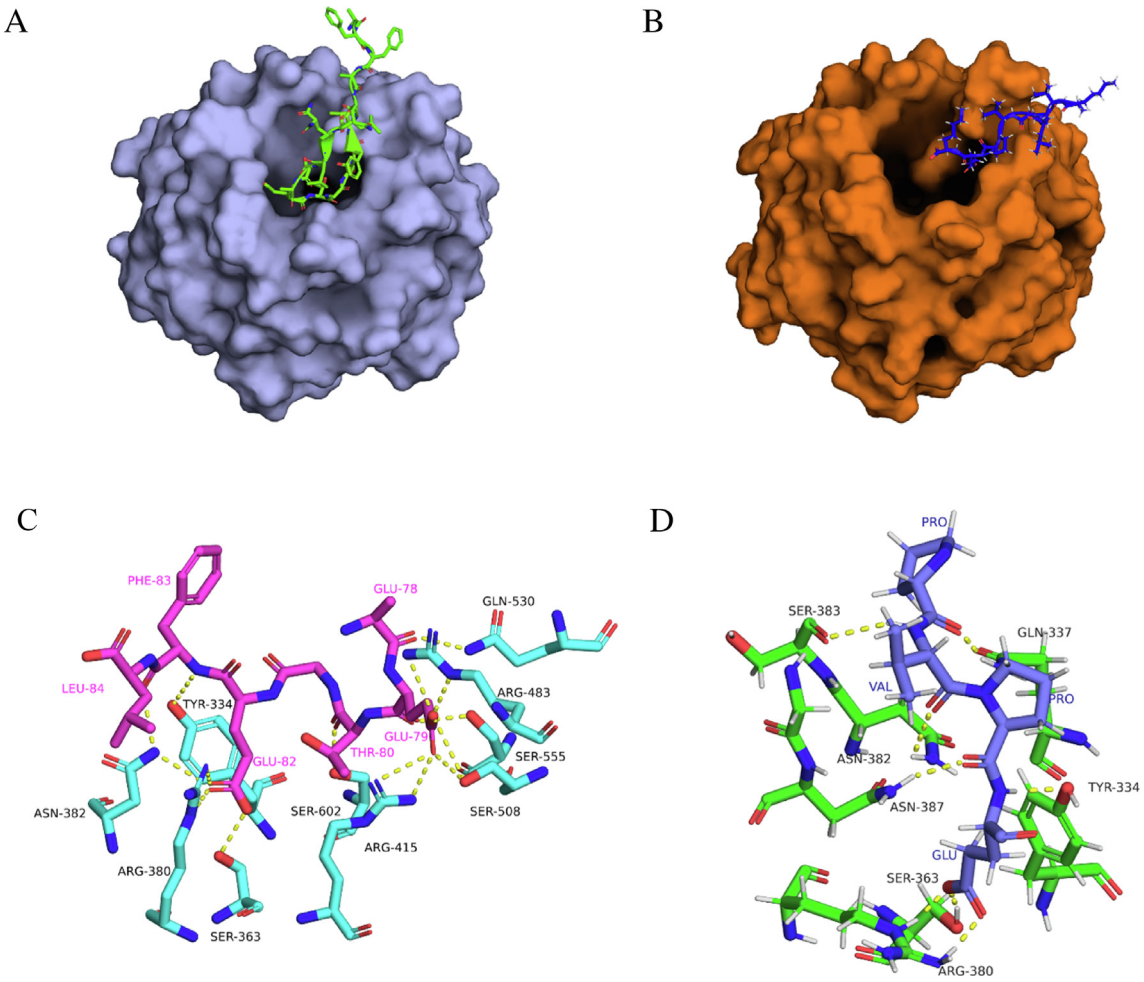

Fig. 5. Comparison between the computational geometries of binding of Neh domain and K-8-K with the pocket of Keap1. **A**: Crystal structure of Keap1 and the Neh domain of Nrf2 (2FLU). **B**: Binding geometry of K-8-K in the pocket of Keap1. **C**: Magnification of the interaction site of Keap1-Nrf2 complex. Hydrogen bonds are indicated with yellow dashed lines. **D**: Magnification of the interaction of Keap1 pocket with K-8-K. Amino acids involved in hydrogen bonds were highlighted: Pro with Gln-337, Val with Ser-383 and Asn-382, Pro with Asn-387, Glu with Tyr-334, Arg-380 and Ser-363.

of Keap1, such as two serines (Ser-363 and Ser-383), two asparagines (Asn-382 and Asn-387) and a tyrosine (Tyr-334). As reported in Fig. 5C, some Keap1 residues that interacted with K-8-K (Ser-363, Arg-380, Asn-382 and Tyr-334) were also involved in the binding of Nrf2 to the Keap1 pocket.

4. Discussion

Based on *in vitro* analyses, peptides N-6-R and A-7-R exhibited some reactivity with the ABTS radical while K-8-K and A-11-M were ineffective (Table S1). Moreover, all four peptides exhibited scarce or null reactivity in comparison to Trolox C, indicating that they are quite ineffective in scavenging free radicals (Fig. S1). In line with the above reported results, peptides A-7-R and N-6-R displayed a relatively small reactivity in crocin bleaching test, while the other two could be considered completely ineffective (see also k_a/k_c values reported in Fig. S1). On the other hand, former observations (Tonolo et al., 2018) have shown that the four peptides were able to prevent cell death caused by oxidative stress, as they prevented the decrease of total thiols and glutathione induced by peroxides. In addition, they protected the activity of thiol-dependent antioxidant enzymes such as thioredoxin and glutathione reductases. In order to evaluate the capability of these compounds to counteract oxidative processes, lipid peroxidation occurrence in Caco-2 cells was analysed as formation of malondialdehyde (MDA), a final product of unsaturated lipid degradation. Cells pre-treated with the four peptides and subjected to oxidative stress through TbOOH action, exhibited a lower amount of MDA formation compared to the control in the presence of TbOOH alone (Fig. 1). In particular, in the presence of N-6-R and K-8-K a decrease up to 30% of MDA production was detected. Together with previous observations (Tonolo et al., 2018) indicating a marked capability of these peptides, K-8-K in particular, of inhibiting ROS production in Caco-2 cells, these results suggest the presence of other mechanisms of antioxidant activity in addition to the direct, although weak, antioxidant scavenging function observed *in vitro*. Given the effect of pre-treatment with peptides on antioxidant enzymes, other pathways eliciting the antioxidant action seemed to be present. Since phase II enzymes were involved, we started to study the Keap1-Nrf2 pathway. Therefore, we investigated if, in Caco-2 cells treated for 24 h with the four peptides, the Nrf2 levels in the nucleus increased as consequence of the activation of Keap1-Nrf2 pathway. Our results showed that the four peptides were able to stimulate the translocation of Nrf2 to the nucleus of the cells, with peptide K-8-K being the most active (Fig. 2). Furthermore, Western blot analysis showed that the four peptides led to the overexpression of antioxidant genes and to the downstream biosynthesis of SOD1, Trx1, TrxR1, GR and NQO1. Notably, K-8-K was the most effective peptide in determining the overexpression of these enzymes (Fig. 3). In addition, the NQO1 and Trx1 enzymatic activities were measured and they matched the overexpression of these antioxidant enzymes, in particular in cells treated with K-8-K (Fig. 4). All these results are in agreement with those

previously reported (Tonolo et al., 2018) regarding thioredoxin reductase and glutathione reductase activities in Caco-2 cells after treatment with these peptides. In order to further evaluate the interaction between K-8-K, the most active peptide, with Keap1-Nrf2, a molecular docking analysis to Keap1 was performed. The results showed that K-8-K was able to interact with key amino acid residues in the same pocket of Keap1 involved in the binding of Nrf2 (Fig. 5C). Our results indicate that the antioxidant properties of the bioactive peptide K-8-K are possibly due to the disruption of Keap1-Nrf2 interaction and to the occupancy of the Nrf2 binding site by K-8-K, which elicits the subsequent activation of Nrf2 signalling pathway.

5. Conclusions

In this work we show that milk protein-derived peptides, potentially able to cross the intestinal barrier (Tonolo et al., 2018), exert an antioxidant action via the activation of the Keap1-Nrf2 signalling pathway. To the best of our knowledge, only a limited number of studies describing the mechanism of action of antioxidant bioactive peptides are available. However, according to these findings there is a general agreement that Nrf2 activation depends not only on the well-known interaction with oxidants and electrophilic molecules, but also on the action of whey proteins and specific peptides (Kerasioti, Stagos, Tzimi, & Kouretas, 2016; Li et al., 2017). Moreover, there are several instances indicating that peptides can reveal their antioxidant effectiveness through induction of phase II detoxifying and antioxidant enzymes by the Keap1-Nrf2 system (Kansanen, Kuosmanen, Leinonen, & Levonenn, 2013; Kim et al., 2013; Li, Li, Li, & Zhou, 2014; Park, Kim, Ahn, & Je, 2016).

As a whole, the obtained results suggest that the synthetic peptides A-11-M, A-7-R, N-6-R, K-8-K, corresponding to milk-derived peptides, are able to protect Caco-2 cells against peroxide-induced oxidative stress by activating the Keap1-Nrf2 pathway and, hence, promoting the expression of the antioxidant enzymes SOD1, Trx1 and TrxR1. Furthermore, *in silico* simulations showed that K-8-K inhibited the interaction between Keap1 and Nrf2, by binding to the site of Nrf2 in Keap1 pocket, with the consequent activation of the cell antioxidant defences.

In conclusion, this work shows that, similarly to what previously reported for other peptides, milk-derived peptides can act by activating the transcription factor Nrf2 and stimulate an increased expression of antioxidant enzymes. This observation can have an impact on further studies evaluating the mechanism of action of various bioactive peptides.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.103696.

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