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# Stability of oligopeptides in solution. Proteolytic digestion and potential dimerization process.

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| **Abstract:** | The specificity of peptide activity depends on the sequence of amino acids and structure, so it is easy to understand the great degree of biodiversity that peptides can cover. Accordingly, the literature shows that peptides carry out various activities, including antiproliferative, antihypertensive, antimicrobial, antioxidant, anticholesterolemic, opioid and antidiabetic activities. However, the potential activity of peptides is strongly quenched by their low bioavailability. The aim of this work is to provide some insights on the possibility of increasing the bioavailability of peptides. Two key points have been investigated. The results demonstrate that N-terminus acetylation and C-terminus amidation can strongly protect peptides from proteolytic degradation. Furthermore, the activity of the peptides can be reduced by the formation of complexes, for example through hydrophobic interactions. In particular peptides containing cysteine can dimerize with the formation of a disulfide bridge. The possibility of decreasing complexes formation by solubilizing peptides in a dissociation mixture (containing glycine, urea, dithiothreitol and β-mercaptoethanol), is discussed. |
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# Stability of oligopeptides in solution. Proteolytic digestion and potential dimerization process.

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**Abstract**

The specificity of peptide activity depends on the sequence of amino acids and structure, so it is easy to understand the great degree of biodiversity that peptides can cover. Accordingly, the literature shows that peptides carry out various activities, including antiproliferative, antihypertensive, antimicrobial, antioxidant, anticholesterolemic, opioid and antidiabetic activities. However, the potential activity of peptides is strongly quenched by their low bioavailability. The aim of this work is to provide some insights on the possibility of increasing the bioavailability of peptides. Two key points have been investigated. The results demonstrate that N-terminus acetylation and C-terminus amidation can strongly protect peptides from proteolytic degradation. Furthermore, the activity of the peptides can be reduced by the formation of complexes, for example through hydrophobic interactions. In particular peptides containing cysteine can dimerize with the formation of a disulfide bridge. The possibility of decreasing complexes formation by solubilizing peptides in a dissociation mixture (containing glycine, urea, dithiothreitol and beta-mercaptoethanol), is discussed.

**Keywords**: Foetal bovine serum, C-terminal amidation, N-terminal acetylation, disulfide bridge, antiproliferative activity.

## Introduction

In the last few decades research has been developed on the properties of natural active compounds. Relations between health and natural active compounds have been developed and strengthened (Eicholzer et al. 2001; Anderson et al. 1994; Ames 1998; Gerber et al. 2002). Among the various classes of active compounds, proteins and peptides are of considerable interest (Mecocci et al. 2000). Taking into account that the specificity of peptide activity depends on the sequence of amino acids and structure, it is easy to understand the great degree of biodiversity that peptides can cover. Consequently, there is an increased interest in peptides in pharmaceutical research and development

and approximately 140 peptides are currently being evaluated in clinical trials (Galdiero and Gomes 2017).

The literature of recent decades shows that peptides carry out various activities, including antiproliferative, antihypertensive, antimicrobial, antioxidant, anticholesterolemic, opioid and antidiabetic activities (Gianfranceschi et al. 2018). The biological and therapeutic properties of peptides can be quenched or modified by their low bioavailability.

Three main factors can influence peptide bioavailability in vivo:

*1) Structural integrity*. The peptide structure can be degraded by exo- and endopeptidase. Protein hydrolysis occurs widely in the body, and especially in the gastrointestinal tract, most peptides show low bioavailability. Furthermore, the formation of peptide complexes due to the dimerization process by disulfide bridges or to hydrophobic interactions must be taken into account.

*2) Cell permeability*. Many peptide structures are poorly permeable to the cell membrane. The binding to peptide N-terminal of cell-permeable motifs has been reported.

*3) Quick elimination*. Oligopeptides can be quickly eliminated by kidney function (Yao et al. 2018).

In our previous paper, the tripeptides AcGly–Phe–Asn(OH) and AcGly–Phe–Asn(NH2) have been tested in vitro for their antiproliferative activity on human breast adenocarcinoma cells (MDA-MB 231) and human dermal fibroblasts (HuDe) (Quassinti et al. 2020). The results show that the peptides significantly affect the proliferation of MDA-MB 231 and HuDe cells in different manner indicating that C-terminal amidation plays a critical role.

Presence of protease in blood that influence the half life times of peptides has been presented in literature (Werle and Bernkop-Schnürch 2006). Example of peptides rapidly degraded by plasma proteases are also reported (Murphey et al. 2000; Zhu et al. 2003). In our in vitro test, the main factor, that determines the peptides stability, was the presence of proteases in the foetal bovine serum.

In this paper we report the results of a research concerning the study of peptide structural stability with reference to proteolytic degradation and potential aggregation process. The experiments were

performed with tri- and pentapeptides designed according to biochemical characteristics of peptides isolated from extracts of broccoli, Brussels and wheat sprouts (Perni et al. 2011).

## Material and Methods

*Chemicals and reagents*

HPLC grade acetonitrile was purchased from Honeywell (Muskegon, MI, USA). Deionized water was prepared in Crystal B30 EDI Adrona deionizer (Riga, Latvia). Peptides utilized for the experimentation represent molecular models of peptide molecules isolated from broccoli, Brussels and wheat sprouts extracts (Perni et al. 2011). Peptides **2**, **3**, and **4** were synthesized by Doctors A. Caporale and M. Ruvo (Istituto di Biostrutture e Bioimmagini, CNR, via Mezzocannone ,16, Napoli 80134, Italy) as described in (Quassinti et al. 2020). Custom designed peptide **1** was purchased from Genscript (Piscataway, New Jersey, USA) at > 94.6 % purity by HPLC. Custom designed peptide **5** was purchased from DBA Italia (Segrate, Milano).

Cell culture media, L-glutamine, penicillin, streptomycin, heat-inactivated foetal bovine serum (HI-FBS), 96-well microtiter tissue culture plates and trypan blue solution were purchased from Corning, Manassas, VA, USA. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were from Sigma-Aldrich, Milano, Italy.

*Peptides stability*

Synthetic peptides were solubilized at the concentration of 10 mM with $H_2O$ (peptide **1**), ethanol (peptide **2** and **4**), DMSO (peptide **3**). These stock solutions were stored at -20°C until use. The determination of peptide stability in HI-FBS was carried out as follow. Synthetic peptides, at the final concentration of 500 µM, were incubated with 10% HI-FBS in PBS solution in a total reaction volume of 200 µl at 37°C. At pre-established time aliquots of 20 µl were withdraw and injected, for

reverse phase HPLC analysis. Chromatographic analysis was performed on Beckman HPLC System Gold Nouveau equipped with a 168 UV detector. A Phenomenex HyperClone $C_{18}$ column (4.6 mm × 250 mm, 5 μm) coupled with a Phenomenex $C_{18}$ guard column (4.0 mm × 3.0 mm, 5 μm) were used for analysis. The mobile phase consisted of solvent A ($H_2O$) and B (MeCN) with a gradient elution as follows: gradient of 0–70% B from 5 to 65 min, then gradient of 70–100% B from 65 to 70 min, isocratic at 100% B from 70 to 80 min, and returning to the initial condition. The flow rate was 0.5 mL/min, and the injection volume was 20 μL. The elution of peptide **1** was performed with 0.1% TFA in $H_2O$ (solvent A) and 0.1% TFA in MeCN (solvent B) with gradient of 0-30% of B in 20 min at flow rate of 1 mL/min.

*HeLa cells survival and viability*

Human uterus adenocarcinoma cells (HeLa, from ATCC cell lines, catalogue number CCL-2), were cultured in Dulbecco's minimum essential medium (DMEM) with 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin and supplemented with 10% heat-inactivated foetal bovine serum (HI-FBS). Cells were grown in a humidified atmosphere with 5% $CO_2$ at 37°C. Cells were maintained in culture by detachment with Tripsin/EDTA and diluted in fresh medium before reaching the cell confluence state (approximately 80% confluence). HeLa cells, in exponential phase of growth, are exposed to different concentration of peptides. After two or three doubling times (48, 72 h) surviving cell number is determined indirectly by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) dye reduction. Briefly, exponentially growing cells were seeded in quadruplicate at the density of $2 \times 10^4$ cells/mL into 96-well microtiter tissue culture plates in a final volume of 200 μL per well of cell culture medium. At selected times (48 or 72 h), 20 μL of MTT solution (5 mg $ml^{-1}$ in PBS) was added to each well and then incubated at 37°C for 3 h. The supernatant was removed and 200 μL of dimethyl sulfoxide (DMSO) was added to each well. The plate was shaken for 5 min and incubated for 30 min at 37°C before being read at 540 nm on a microplate spectrophotometer FLUOstar Omega (BMG Labtech). Cell number was determined

from the obtained optical density values using a standard curve. The trypan blue exclusion assay was performed by staining cells with trypan blue (0.5% in PBS) and counting in a Burker camera. Blue stained cells were considered nonviable.

*Mass spectrometry analysis*

Lyophilized samples of peptide fraction, isolated from broccoli, Brussels or wheat sprouts and purified by gel filtration and HPLC, were solubilized in methanol and injected for electrospray mass spectrometry analysis. Mass spectrometer: LCQ-MS THERMOQUEST/ESI-ION TRAP. Capillary temperature 220°C, capillary voltage 10V, spray voltage 4 kV and collision energy from 17 to 23 keV. The mass spectra analysis was performed with a recently reported automatic combinatorial method that carries out the computation of all amino acid sequences compatible with a given molecular ion. The computation has been performed by utilizing both the monoisotopic and the average isotopic molecular weight of the amino acids (Bruni et al. 2005). The possible sequences of these compounds are automatically obtained by considering the mass of ions that are potential breakdown products (Sadagopan and Watson 2001, Bruni et al. 2005).

**Results**

Peptides **1**, **2**, and **3** (Table 1) represent molecular models of compounds isolated from broccoli sprouts (**1**) and Brussels sprouts (**2**, **3**), respectively. Recently we reported that AcGly-Phe-Asn(OH) (peptide **2**) and AcGly-Phe-Asn($NH_2$) (peptide **3**) tripeptides selectively affect the proliferation rate of MDA-MB 231 and HuDe cells (Quassinti et al. 2020). Tripeptides **1**, **2**, and **3** were analyzed for their susceptibility to proteolytic degradation by foetal bovine serum proteases. Peptides were incubated at 37°C in presence of 10% of HI-FBS, the same concentration of serum present in the cell culture medium used in in vitro tests and the hydrolytic products were analyzed by HPLC. The results, reported in Fig. 1, show peptide **1** (16.317 min elution time) after 6 hrs of incubation in presence of 10% of HI-FBS. The peptide, that presents free amino- and carboxy-terminal, is

completely hydrolyzed giving two products with elution time of 14.717 min and 16.817 min (Fig.1b). On the contrary, peptide **2** (26.667 min elution time), that presents acetylated N-terminal showed a lesser hydrolysis after 24 h of incubation in presence of HI-FBS (Fig.1d). Two peptide products, with elution time of 24.133 and 27.137 min, were derived from the hydrolysis of peptide **2** corresponding at a hydrolysis of 25% of initial peptide calculated by the ratio between peak areas of integral peptide before and after incubation time. Once more, peptide **3** (26.883 min elution time), with acetylated N-terminal and amidated C-terminal, appears much more resistant to hydrolytic degradation even after 24 h of incubation in presence of HI-FBS (Fig. 1f).

The resistance to proteolytic hydrolysis of pentapeptide **4** (Table 1) with blocked N- and C-terminals has been subsequently measured. Also the pentapeptide with blocked terminals is almost completely resistant to degradation by serum proteases (Fig. 2 a, b). Although, the HPLC elution profile of peptide **4** (25.217 min.) shows, following 24 hr of incubation, a product with elution time of 27.017 min that probably corresponds to dimerization of the containing cysteine peptide with the formation of a disulfide bridge. This hypothesis is confirmed by the observation that the peptide dimerization can be reversed by adding beta-mercaptoethanol at the final concentration of 1 mM and incubated further 1 h and then injected for the HPLC analysis (Fig. 2 c). Indeed, the peak eluting at 26.967 almost completely disappears and appears a peak at 25.100 min corresponding to integral peptide **4**. The other peak with elution time of 26.217 min probably corresponds to combination of peptide **4** with beta-mercaptoethanol that indeed presents an elution time of 14.817 min.

For what concerns the peptide **5** (Table 1) isolated from wheat sprouts fraction, it was subjected to extensive mass spectrometry analysis (MS/MS; MS/MS/MS; MS/MS/ MS/MS) according to following scheme:

MS N- ----------572 $MH^+$----------C

$MS^2$ of 572 ion N------ A1---- CO--NH----------C

≠←----------m/z 391+$2H^+$ frag y4----------C

$MS^3$ of 393 ion N--------A1--------A2------CO--NH----------C

≠←----------m/z 348 frag.x3----------C

$MS^4$ of 348 ion │------A3-------A4------CO--NH------A5---C

│---m/z 245 int.frag..----→≠

│---m/z 260 int.frag.----------→≠

The results of this analysis appear consistent with the sequence AcHis-Ala-Asn-Cys-Ser-$NH_2$ that was synthesized.

At the same time the wheat sprouts purified fraction utilized for the peptide isolation and structural study was subjected to incubation at 45°C for 36 hrs, with or without the presence of 25% ammonia, in order to check the possibility of peptide glycosylation (Haynes and Aebersold 2000). The results exclude this hypothesis. Moreover, the mass spectrometry analysis of the fraction incubated at 45°C for 36 hrs also in absence of 25% ammonia shows that the ion m/z =572 is not detectable. Vice versa the treatment produces an ion m/z =1143 ($1142+H^+$). The sequence of peptide 5 containing cysteine could be in agreement with the formation of a peptide dimer. Any way if the dimerization is due to a disulfide bridge between cysteine the expected mass value should be $[(571x2) – 2]H^+ = 1141$. The possibility of a peptide dimer caused by hydrophobic bond should be taken into consideration.

The peptide **5** causes a sharp dose-dependent inhibition of HeLa cell proliferation (70% and 90% inhibition with 25 ng and 50 ng/ml, respectively). The treatment at 45°C for 36 hrs causes a strong reduction of antiproliferative activity (from 90% to 30% inhibition with 50 ng/mL peptide). Consequently, the peptide dimerization causes a significant inhibition of peptide activity.

**Discussion**

The sensitivity of peptides **1**, **2**, and **3** to proteolytic digestion was measured following incubation with foetal bovine serum. The incubation mixture was subjected to HPLC chromatography to check

the formation of compounds with elution time different respect to elution time of the whole molecule. The results show that peptide **1** is degraded after 6 hrs of incubation. Peptide **2** (N-terminal acetylated) and peptide **3** (N-terminal acetylated and C-terminal amidated) are almost completely protected from proteolytic digestion. This results have been confirmed also with pentapeptide **4** (N- and C-terminals blocked). These data are in agreement with the results reported by Brinckerhoff et al. (1999) which demonstrate that C-terminal amidation and /or N-terminal acetylation markedly increases the stability of peptide AAGIGILTV incubated in plasma obtained from human blood. Moreover, some tripeptides involved in the control of cell proliferation have also been considered, among them the melanocyte inhibitor peptide, pyroGlu-Phe-Gly($NH_2$) (Gembitsky et al. 2000); the colon mitosis inhibitor peptide, pyroGlu-His-Gly(NH2) (Reichelt et al. 2004); and the T-lymphocyte inhibitor peptide, AcGlu-Ser-Gly($NH_2$) (Liu et al. 2003). These peptides show the block of N-terminal groups (N-acetylation or presence of pyroGlu) and C-terminal groups (C-amidation), features that can prevent peptide hydrolysis by digestive proteases. The pentapeptide pyroGlu-Glu-Asp-Ser-GlyOH inhibits proliferation and enhances terminal differentiation in cultured mouse epidermal cells (Elgjo et al. 1986). Despite the presence of pyroGlu at N-terminal this peptide is sensitive (available) to attack by serum proteases. It has been reported that synthetic epidermal pentapeptide can be phosphorylated in vitro at serine level by protein casein kinase II isolated from calf thymus chromatin. The phosphorylated form of pentapeptide is protected from the serum protease activity (Bramucci et al. 1992).
Similarly, the clusters of phosphorylated serine residues of caseinophosphopeptides (Ser(P)-Ser(P)-Ser(P)-Glu-Glu) prevent their hydrolysis by digestive proteases, protect their mineral-binding capacity, and give them a better chance of being absorbed (Boutrou et al. 2008; Perego et al. 2012).
The experiments performed with peptide **4** (Table 1) following the HPLC chromatography, show the formation of a compound more hydrophobic than the starting peptide. The hypothesis is the formation of a dimer, probably trough a disulfide bridge because the peptide has a cysteine

residue (Mthembu et al. 2020) This hypothesis is confirmed because the dimer formation may be reversed by the presence of 1 mM of beta-mercaptoethanol.

The peptide **5** (Table 1) was isolated from wheat sprouts extract. The mass spectrometry of the purified fraction shows a main molecular ion with m/z = 572 $MH^+$. This fraction exerts a strong inhibition of the HeLa tumor cells proliferation (70% inhibition at peptide 25 ng/mL and 90% inhibition at 50 ng/mL). To study the structure of this fraction we have also checked if the peptide molecule is glycosylated. Consequently, the fraction was subjected to a beta-elimination treatment that causes the dissociation of O-linked sugars. So the fraction was treated at 45 °C for 36 hrs with or without 25% ammonia. The antiproliferative activity is strongly inhibited by incubation at 45°C for 36 hrs also in absence of ammonia (from 90% to 30% inhibition at peptide 50 ng/ml). At the same time the mass spectrometry analysis of ions of second, third and fourth generation indicated the sequence AcHis-Ala-Asn-Cys-Ser-$NH_2$ that was synthesized. In the fraction treated at 45°C for 36 hrs the ion m/z 572 $MH^+$ is practically no detectable; on the contrary a main ion with m/z 1143 $MH^+$ is generated by the treatment. The 1143 $MH^+$ ion can correspond to a dimer of 572 $MH^+$ ion. This possibility is supported by the presence of a cysteine residue in the peptide structure. However, a potential dimer caused by a disulfide bridge should have a m/z = 1141 (not 1143). Probably the dimer is due to a specific probably hydrophobic interaction. These data are in agreement with the results reported by Arnone et al. (1995) according to which the DNA binding activity of the thyroid transcription factor (TTF-1) is inhibited by oxidation due to formation of disulfide bonds. Accordingly, this inhibition is fully restored upon exposure to 50 mM dithiothreitol.

In addition, Blank et al. (2000) described experimental conditions to prevent the dimerization of containing cysteine peptides. The activity of peptides 29-35 and 122-139 from human interferon alpha-2 (IFN-α) was studied and to prevent the formation of dimers and aggregates they were solubilized by a dissociation medium containing glycine, urea, dithiothreitol and beta-mercaptoethanol.

In conclusion this paper show results of research directed to increase the stability of oligopeptides in solution. This is important also for the potential use in nutraceutical field. First, the stability of oligopeptides respect to proteolytic degradation is markedly increased by the block of N- and C-terminals. Second, the experiments performed with peptides **4** and **5** demonstrated by means of HPLC chromatography and mass spectrometry, respectively, the formation of dimers (or aggregates). To avoid the formation of dimers and/or aggregates the purified or synthesized peptides should be solubilized in a dissociation mixture.

**Declarations**

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Compliance with ethical standards: no.

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Data availability statements: Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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Figure Captions

**Fig 1** Reverse phase HPLC profiles of peptide **1**, **2**, and **3** incubated at 37°C in presence of 10% of heat-inactivated foetal bovine serum. a) and b) represent peptide **1** at 0 and 6 h of incubation time; c) and d) peptide **2** at 0 and 24 h; e) and f) peptide **3** at 0 and 24h (the big peak eluting at 9 min represent the DMSO solvent)

**Fig 2** Reverse phase HPLC profiles of peptide **4** incubated at 37°C in presence of 10% of heat-inactivated foetal bovine serum. a) and b) represent peptide **4** at 0 and 24 h of incubation time; c) represents elution profile of peptide **4** treated for 24 h in presence of 10% HI-FBS and 1 h of β-mercaptoethanol

Fig 1

Click here to access/download;line figure;Fig 1.tif

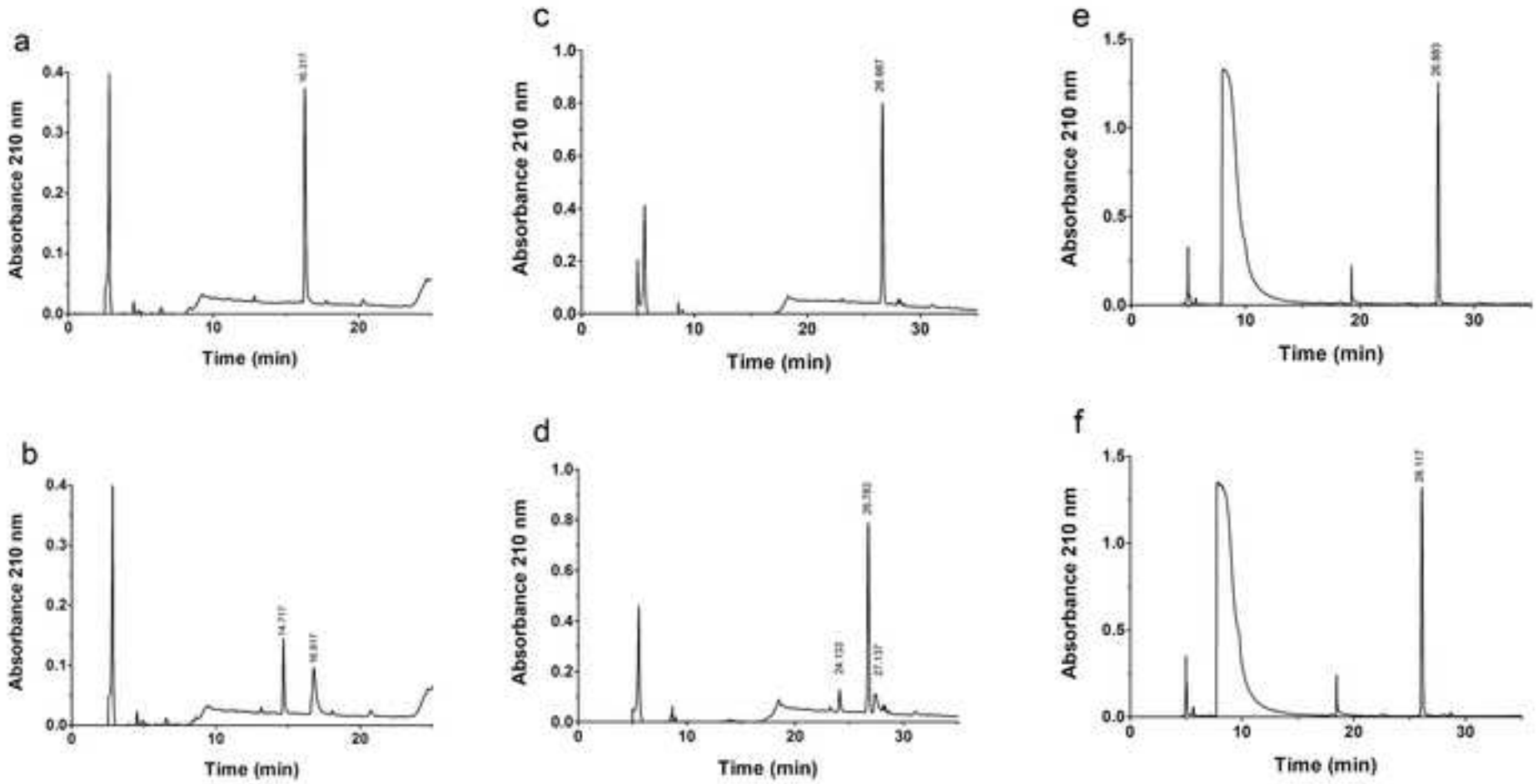

Fig 2

Click here to access/download;line figure;Fig 2.tif

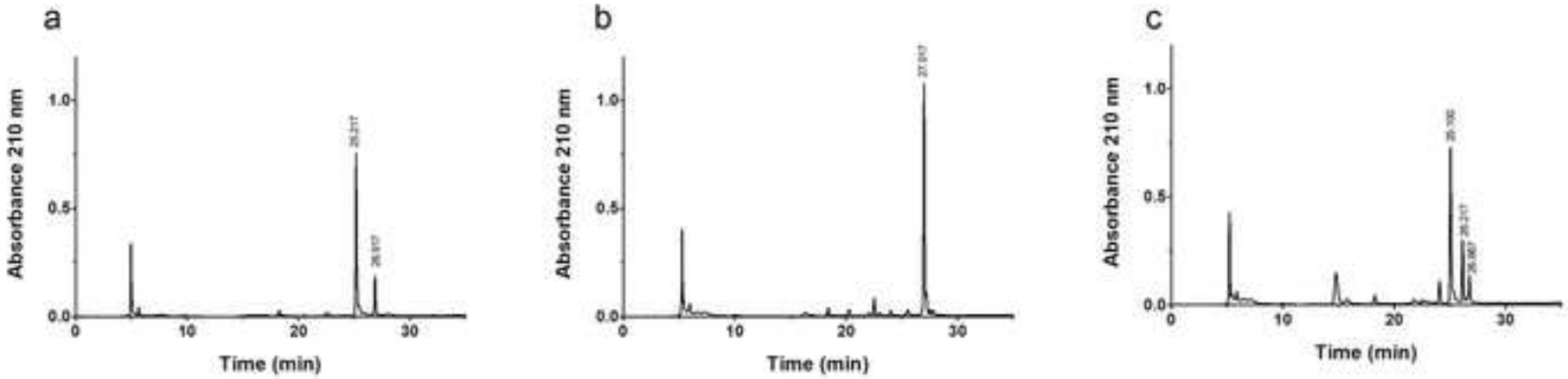

**Table 1** Peptides utilized for the experimentation represent molecular models of peptide molecules isolated from broccoli, Brussels and wheat sprouts extracts

| **Peptide** | **Ion $MH^+$** | **Considered ion fragments** | **Sequence consistent with mass spectrometry** |
|---|---|---|---|
| 1[a] | 310.2 | 58.1: $b_1$; 251.3: $y_2$; 147.2: i.f. $b_1$-$y_1$ | H-Gly-Phe-Ser-OH |
| 2[b] | 379.4 | 132.7: $y_1$; 219.2: $a_2$ | AcGly-Phe-Asn-OH |
| 3[b] | 378.3 | analog of peptide **2**- | AcGly-Phe-Asn-$NH_2$ |
| 4[c] | 541.0 | 364.3: $b_3$; 203.2 : i.f. $a_2$-$z_1$; 178.2: $y_2$ | AcThr-Tyr-Gly-Cys-Gly-$NH_2$ |
| 5[c] | 572.1 | 393.0: $y_4$ ; 348.1: $x_3$; 260.1: i.f. $a_2$-$z_1$; 245.4: i.f. $a_2$-$y_1$ | AcHis-Ala-Asn-Cys-Ser-$NH_2$ |

[a]from broccoli sprouts i.f. = internal fragment

[b]from Brussels sprouts

[c]from wheat sprouts

The fragments utilized for the design of peptide sequences were deduced from $MS^2$, $MS^3$ and $MS^4$ spectrometry analysis of the main ions observed in the mass spectra. The N- and C-terminal fragments are indicated following the international nomenclature (Roepstorff and Fohlman 1984; Sadagopan and Watson 2001). Peptides **2**, **3**, and **4** were synthesized by Doctors A. Caporale and M. Ruvo (Istituto di Biostrutture e Bioimmagini, CNR, via Mezzocannone ,16, Napoli 80134, Italy). Peptides **1** and **5** were obtained by custom synthesis