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Antioxidant, antityrosinase and antibiofilm activities of synthesized peptides derived from *Vicia faba* protein hydrolysate: A powerful agents in cosmetic application

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ABSTRACT

The aim of this study was to identify peptides with antioxidant, antityrosinase and antibiofilm activities released from *Vicia faba* seed proteins hydrolysate. The hydrolysis pattern was performed with trypsin. Firstly, the obtained hydrolysate was fractionated by cation exchange chromatography and the fraction exhibiting the highest biological activity was then analyzed by reverse phase HPLC tandem mass spectrometry. After annotation by Peaks software (BSI, Canada) for *de novo* peptide sequencing, seven peptides were identified, mainly derived from storage proteins: legumin and vicilin. These peptides were further chemically synthesized to assess their antioxidant capacity, antityrosinase activity and antibiofilm ability against *Pseudomonas aeruginosa* PA14. Results showed that peptides P5, P6 and P7 identified as LSPGDVLVIPAGYPVAIK, VESEAGLTETWNPNHPELR and EEYDEEKEQGEEEIR respectively, displayed the highest DPPH radical scavenging activity (IC_{50} = 0.25–1.9 mM). P5 was the only peptide able to chelate iron and reduce Fe^{3+} to Fe^{2+}. Furthermore, peptides P4 (GPLVHPQSQSQSN) and P6 were observed as potent tyrosinase inhibitors with IC_{50} values of 1 and 0.14 mM respectively. P1, P5, P6 and P7 also showed interesting antibiofilm activity against *Pseudomonas aeruginosa* PA14 with $MBIC_{50}$ value ranged from 12 to 35 μM. Our data suggest that *V. faba* seed proteins hydrolysate could be potentially used as a source of natural bioactive peptides for cosmetic and pharmaceutical applications.

1. Introduction

Faba bean (*V. faba*) is a broadly used legume in the Mediterranean region as a protein source for both human and animal feeding. Its high content in proteins (25% to 35%) and other essential nutrients (lecithin, choline, dietary fiber, minerals, and complex carbohydrates) (Hedley, 2001) make this legume a precious substitute to animal protein in human diet, especially in low-income countries. It is also noteworthy that *V. faba* has been suggested to be beneficial in the treatment of Parkinson disease (Rabey et al., 1992), hypertension, renal failure and liver cirrhosis (Vered et al., 1997). However, despite its high value food product and its richness in proteins, bioactive compounds isolated from *V. faba* seed proteins have been only poorly investigated so far. In recent years, bioactive peptides released by enzymatic digestion of dietary proteins from legumes grains are gaining strong interest because they have been used as opiates, immunomodulators, anticarcinogens, antimicrobials, antioxidants, antihypertensives and antithrombotic agents (Duranti, 2006). Among the numerous bioactive peptides, antioxidant peptides have been stated as powerful agents against oxidative damage by free radicals or reactive oxygen species (Zhang et al., 2009). At excessive amounts, these reactive species can cause several health diseases including cancer, diabete, atherosclerosis, coronary heart disease, neurological malfunctioning or weakening of the immune system (Comfort et al., 2011). Natural antioxidant peptides have received more

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attention because they can be used at higher concentrations without the toxic side effects in comparison with synthetic antioxidant compounds (Pownall et al., 2010). The underlying mechanism of action may be scavenging free radicals and/or chelation of transition metals (Pihlanto-Leppala, 2006). Antioxidant abilities of the peptides are thought to be due to their sequence, amino acid composition and hydrophobicity (Sarmadi and Ismail, 2010).

In the other hand, Jiménez-Cervantes et al. (2001) have reported that free radicals also up-regulate the mRNA level for tyrosinase, and amplified the production of melanin to possess serious problems in human skin such as melasma, freckles, age spots and malignant melanomas (Chang et al., 2013). Since, tyrosinase is the key enzyme catalysing melanin biosynthesis and is highly associated with pigmentation disorders, development of tyrosinase inhibitors are of great importance in cosmetics and in medication. There are many tyrosinase inhibitors known today such as hydroquinone, kojic acid, azelaic acid, electron-rich phenols and arbutin, but their use is still limited due to their adverse side effects like skin irritation, toxicity toward melanocytes cells and low stability (Chen et al., 2015). Therefore, there is a need to screen for natural and safe compounds. Recently, tyrosinase inhibitors from natural sources such as proteins and peptides from milk, wheat and silk have been reported (Schurink et al., 2007).

Pseudomonas aeruginosa, an opportunistic human pathogen, represents a vital cause of nosocomial and life threatening infections of immunocompromised patients. It is one of the main causes of chronic wound infections that often become colonized with a range of other bacterial species like *Staphylococcus aureus* and *Enterococcus faecalis* (Rybtke et al., 2015). *P. aeruginosa* can colonise human body tissues and medical devices (Kim et al., 2015) by forming a biofilm in which bacterial cells are encased in a self-produced biopolymer matrix composed by proteins, polysaccharides, DNA and lipids (Bjarnsholt et al., 2013). It was reported that biofilm cells are more resistant to conventional antibiotics and host immune response. Therefore, the search of alternative antibiofilm therapeutics has become urgently needed. Antibiofilm peptides represent a promising approach to treat biofilm-related infections (Pletzer et al., 2016). With increasing demand for natural bioactive compounds in cosmetic and healthcare industries, we addressed in the present study the identification of peptides released from *V. faba* seed proteins hydrolysate and the evaluation of their antioxidant, antityrosinase and antibiofilm activities against *P. aeruginosa* PA14.

2. Materials and methods

2.1. Materials

V. faba seeds used in this study were purchased from a local market in Tunis (Tunisia). Trypsin, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt (ferrozine), trichloroacetic acid (TCA), iron(II) chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$), iron(III) chloride ($FeCl_3$), 1,4-Dithiothreitol (DTT), calcium chloride ($CaCl_2$), ethylenediaminetetra-acetic acid (EDTA), acid ascorbic, sodium dodecyl sulfate (SDS), L-3,4-dihydroxyphenylalanine (L-DOPA), L-tyrosine and Tyrosinase were purchased from Sigma Aldrich. All other chemicals used were of the highest grade commercially available.

2.2. Extraction and quantification of total proteins

After *V. faba* seeds were lyophilized and grounded, the resulting bean flour was defatted with hexane (1/4, w/v) and then stored at −20 °C until use. Defatted flour was homogenized in an extraction buffer (0.1 M Tris-HCl, pH 8 at 1/5 w/v ratio) and further stirred at 4 °C for 4 h. The homogenate was clarified by centrifugation (12,000 × *g*, 4 °C, 15 min) and the resulting supernatant containing soluble proteins was precipitated with cooled acetone. After centrifugation 10 min at 13,000 × *g*, the supernatant was discarded and the protein pellet was dried in Speed-vacuum. The procedure was repeated three times before the resulting pellet was frozen until further use. The total protein content was determined by Qubit protein assay kit using the Qubit 2.0 fluorometer (Ben Slimene et al., 2012).

2.3. Preparation of V. faba seed proteins hydrolysate (VFSPH)

V. faba seed proteins hydrolysate was prepared according to Kinter and Sherman (2000) with some modifications. *V. faba* seed proteins (VFSP) were dissolved at 2% (w/v) ratio in 50 mM Tris-HCl buffer (pH 7.5) containing 0.1% (w/v) SDS and 5 mM DTT. The protein solution was heated for 15 min in a boiling water bath and then ice-cooled. Trypsin was dissolved in digestion buffer (50 mM tris-HCl, pH 7.5 containing 20 mM $CaCl_2$) and added to the above cited mixture at an enzyme/substrate ratio of 1/20. The hydrolysis was performed overnight at 37 °C. After digestion, trypsin reaction was inactivated by adding 10% (w/v) TCA and the solution was centrifuged at 10,000 × *g* for 5 min. The supernatant was then dialyzed against water, freeze-dried, and stored at −20 °C.

2.4. Determination of the degree of hydrolysis (DH)

The Degree of hydrolysis (DH) is defined as the percentage of the cleaved peptide bonds (Alder-Nissen, 1979). DH of VFSPH was determined according to Pericin et al. (2009) with some modifications. 20% (w/v) TCA was added to an equal volume of hydrolysate and kept for 30 min at 4 °C. This mixture was then centrifuged at 12,000 × *g* for 10 min. The obtained 10% TCA-soluble proteins of the supernatant and hydrolysate mixture (without addition of TCA) were each assayed for their protein content according to Ben Slimene et al. (2012). The DH was calculated as the ratio of 10% TCA-soluble protein to total protein content in the supernatant of hydrolysate mixture, expressed as a percentage.

2.5. Purification of peptides by strong cation exchange chromatography

The lyophilized hydrolysate was dissolved in 25 mM ammonium acetate buffer (pH 3) and fractionated on a strong cation exchange (SCX) column previously equilibrated in the same buffer. Elution of fractions was provided by a discontinuous gradient of increasing pH (3, 4, 5, 6 and 8). All fractions recovered were then lyophilized to be tested for DPPH radical scavenging assay, antityrosinase ability and antibiofilm activity. The most active fraction F1 was collected for further purification.

2.6. Identification of peptides by Orbi-trap

The active fraction obtained from SCX column was subsequently subjected to LC–MS/MS performed with a LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific) equipped with a nano-ESI source. To annotate peptide sequences, MS/MS spectra were analyzed by the Peaks software (BSI, Canada) for *de novo* peptide sequencing. Then, *de novo* results were submitted to MS-BLAST searches (http://genetics.bwh.harvard.edu/msblast/).

2.7. Peptides synthesis

Seven peptides identified in F1 fraction were chemically synthesized by PRIMACEN, IRIB, Rouen, France. The purity of the synthesized peptides was verified by analytical C18 RP-HPLC and their molecular masses determined by MALDI-TOF mass spectrometry.