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Protein hydrolysates from anchovy waste: purification and chemical characterization

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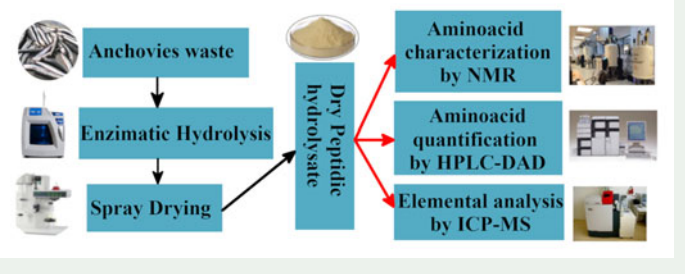
ABSTRACT

The aim of this study is to prompt the recovery of industrial by-products through the production of new functional foods; it takes advantage from new throughput technologies with low environmental impact and high economic sustainability. In the field of fish processing, in order to recover the worthy protein-rich fish waste, residues from the production of Anchovies (*Engraulis encrasicolus*) have been converted into hydrolysate through enzymatic treatment. The obtained hydrolysate product showed a promising biological and nutritional content made by differently sized peptides and free amino acids endowed with assessed benefic effects. The study showed the possibility to produce a dry powder with an activity water (aw) of 0.3–0.5 and an essential amino acids (EAA) fraction of 42.0% over the total amino acids (TAAs). These results pave the way to the smart recovery of commercial products featured by high nutritional value, either as stand-alone items or as components of functional foods.



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1. Introduction

Currently, smart food waste throughput is attracting a great interest, as a growing global issue. The management of food waste includes several sectors spanning from collection, storage and transport to treatment and disposal. EU waste management policy aims to the reduction of environmental and health impacts and to improve Europe's resource efficiency; the enhancement of recycled materials will reduce the

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global intake of natural resources (EU Commission 2018). A smart waste management, pursued by recovering chemicals as fuel, food, nutraceutical or feeding products has become interesting and promising from an economically and environmental point of view (Gervasi et al. 2018a) and could represent a key element in ensuring resource efficiency and sustainable growth economies. From food waste materials, large numbers of interesting biomolecules could be obtained and extracted (Tropea et al. 2013; Alesci et al. 2015; Gervasi et al. 2018b). Due to the growth of fish processing industry, a rising amounts of offal and other by-products are produced (FAO 2018). In the past, fish industry by-products were considered just as waste representing an environmental contamination source. Nowadays fish by-products are gaining increasing interests (He et al. 2013) because of the possible conversion and use as feed, dietetic foodstuffs, pharmaceuticals, nutraceuticals and cosmetics, but also as biofuel production (Piovesana et al. 2018). The major by-products yield from fish processing include viscera, skin, scales, bones; these constitute up to 70% of the original raw material (Benjakul and Morrissey 1997). Fish viscera constitute approximately 20% of the fresh fish biomass and are a rich source of protein and polyunsaturated lipids. These low quality raw materials or wastes if not utilized may cause environmental, health and economic problems (Vidotti et al. 2003). In order to add value to proteinaceous fish waste, specific conversion treatments were used to obtain the so called hydrolysates (Aspmo et al. 2005; He et al. 2014). This process solubilize the protein sources enhancing the biological and nutritional value of the product improving its commercial interest (Nilsang et al. 2005; Villamil et al. 2017). Protein hydrolysis consists of the cleavage of peptide bonds through addition of a water molecule; it leads to the obtainment of free amino acids and low molecular weight peptides (Rutherfurd and Gilani, 2009). Hydrolysis catalyzed by strong acids or bases has been considered as the first choice for the industry, because it is a quick, simple and low cost procedure easily applicable at the industrial scale (Gao et al. 2006). On the other hand, it often have led to low quality products as many amino acidic constructs turned out to be destroyed (Pasupuleti and Braun, 2010; Kristinsson and Rasco, 2000). Enzymatic hydrolysis lead to an interesting peptide and amino acid rich product with high nutritional value and bioactive proprieties (Tavano, 2013; Benjakul et al. 2014); indeed differently sized peptides and combinations of individual amino acids have been shown to perform desirable functions for different purposes (Pasupuleti and Braun, 2010; Benjakul et al. 2014). The main goal of this study was the chemical characterization of the hydrolysates coming from a specific targeted enzymatic treatment of Anchovies' (*Engraulis encrasicolus*) viscera followed by the spry dry processing. In order to define the amino acidic content of the product, we have employed the high performance liquid chromatography with diode array detection (HPLC-DAD) and the liquid nuclear magnetic resonance (NMR) analyses. To monitor the presence of inorganic ions, the inductively coupled plasma mass spectrometry (ICP-MS) was used.

2. Results and discussion

According to He et al. 2013b, we have adopted the enzymatic approach for the treatment on the anchovies' viscera which are by-products coming from the fish industry. In order

Table 1. Amino acids composition of the anchovy protein hydrolysates analyzed by HPLC and NMR. Results are rather consistent demonstrating a worthy amino acidic concentration.

Amino acids	Symbol	measured μM concentration by NMR	Amino acid (g/100g) by HPLC	weight ratio according to NMR analysis	Relative weight by NMR %	Relative weight by HPLC %
Isoleucine	ILE	0.99 ± 0.03	3.26 ± 0.07	1.31	3.03	3.63
Leucine	LEU	2.50 ± 0.07	6.87 ± 0.07	3.28	7.60	7.65
Lysine	LYS	3.63 ± 0.07	10.94 ± 0.05	5.31	12.31	12.18
Methionine	MET	0.86 ± 0.03	2.56 ± 0.02	1.29	2.99	2.85
Phenylalanine	PHE	2.08 ± 0.08	6.83 ± 0.05	3.43	7.97	7.61
Threonine	THR	0.98 ± 0.03	2.42 ± 0.02	1.17	2.71	2.69
Valine	VAL	1.84 ± 0.06	4.19 ± 0.04	2.16	5.00	4.67
Arginine	ARG	2.59 ± 0.05	8.83 ± 0.05	4.52	10.49	9.83
Glycine	GLY	7.12 ± 0.14	10.87 ± 0.03	5.35	12.41	12.10
Proline	PRO	2.48 ± 0.07	5.86 ± 0.05	2.85	6.61	6.53
Tyrosine	TYR	0.76 ± 0.03	3.34 ± 0.02	1.38	3.21	3.72
Alanine	ALA	6.32 ± 0.13	12.06 ± 0.04	5.63	13.05	13.43
Glutamic acid	GLU	3.70 ± 0.11	11.77 ± 0.09	5.44	12.61	13.11
OTHER		–	10.20			
TOTAL		35.86	100.00	43.10	100.00	100.00

to stabilize the resulting product and delay its decomposition, in our laboratories we have used the spray drying procedure. Spray dryer is a smart low-cost instrument converting liquid food products into high quality dry powders (Silva et al. 2011). The amino acidic dry yield ranged between 45 and 54% witnessing an efficient powder recovery in a laboratory scale according to Bhandari et al. (1997). The activity water (aw) of the powder was between 0.3 and 0.5; this range represents a good condition for the product storage. The detected amino acid composition is reported in Table 1. The total content of the EAA was high, and it accounted for approximately 42.0% of the total amino acids (TAAs) and the total percentage of proline, leucine, alanine, and aromatic amino acids was 38.0%. According to Byun et al. (2009), peptides made by these last amino acids displayed a better scavenging effect upon the biologically dangerous free radicals.

The analytical strategy herein used is the crossfire of two quick and smart analytical methods (NMR and HPLC) for the amino acids determination. NMR analysis, empowered by the novel algorithm based on the multi assignment approach MARA-NMR (Rotondo et al. 2019), takes advantage from the ease of sample preparation and data retrieving. As any chemical group of any compound gives the same NMR instrumental response, the relative concentration of amino acids can be measured without any reference standard with an acceptable degree of confidence (Table 1). Moreover the signal assignment of any amino acid in the prepared solutions is well known (see supplemental material), so that MARA-NMR quantification is definitely suitable for our analytical purposes. In this respect, HPLC turns out to be a more troublesome technique requiring standard calibration curves and instrumental optimizations; on another hand, it is known to be a quite precise, common and accurate method. Data in Table 1 shows an acceptable fitting between the two analytical methods prompting next further uses of such paired analyses.

The amounts of major and trace elements, including heavy metals, in the fish powders were determined using ICP-MS (Table 2), one of the most sensitive analytical techniques for the rapid and reliable determination of inorganic elements (Aghraz et al. 2019).

Table 2. a) Major, essential trace element and b) heavy metals composition of anchovy protein hydrolysates revealed by ICP-MS. Levels of major and trace essential elements are expressed as mg kg^{-1}.

a)									
Mg	Al	V	Mn	Fe	Zn	Se	Sr	Ag	Sn
1611.36 ± 45	13.48 ± 1.1	0.36 ± 0.2	0.51 ± 0.1	24.62 ± 1.0	27.00 ± 8.06	0.82 ± 0.05	173339.40	0.02 ± 000.1	0.62 ± 0.1
b)									
Cr	Co	Ni	As	Cd	Pb	Hg			
0.31 ± 0.1	0.04 ± 0.01	0.55 ± 0.2	2.70 ± 0.1	0.04 ± 0.01	0.25 ± 0.05	0.02 ± 0.01			

Beyond the expected general profile concerning the elemental quantification (Table 2), the elements considered as a potential risk for the human health (Cd, Pb, Hg) are far below the limits imposed by the last European law for food integrators (Commission regulation (EC) No 629/2008). This is a very important issue for the possible commercial promotion of the studied product.

The analyses on the fish protein hydrolysate powder highlight the chance of using fish protein hydrolysate as promising high added value products, that, thanks to their nutritional content and bioactive and functional properties, could be applied in the food and pharmaceutical industries, or as microbial growth media, as demonstrated for other agroindustry waste (Gervasi et al. 2019).

3. Experimental

3.1. Materials and chemicals

Anchovies (*E. encrasicolus*) viscera were purchased from the local market Original Giuseppe Curreri Srl in Agrigento, Italy. Fresh fishes after capture were frozen (−20 °C) and stored in polyethylene bags until use. Protamex, Flavourzyme 500MG, Neutrase 0.8 L, and Alcalase 2.4 L were obtained from Novozymes China Inc. (Guangzhou, China). All other chemicals were of analytical grade from Sigma-Aldrich.

3.2. Enzymatic hydrolysis

The frozen anchovies were thawed and ground into mince, then mixed with distilled water at a ratio of 1:3 (w/v). After pH adjustment, with a 2 M sodium hydroxide solution, the commercial proteases were added. The concentrations of the various commercial proteases were 3% (E/S, w/w). The composition of commercial proteases was 1.1:1.0:0.9 for Protamex: Flavourzyme 500MG: Alcalase 2.4 L (Novozymes China Inc.) respectively. The mixtures were incubated in a 5 L bioreactor (BIOSTAT® B Sartorius, Italy) with a working volume of 1 L in batch mode. The bioreactor was operated with strictly controlled parameters: the pH of 7.5 was maintained through the automatic addition of 1 M NaOH and 1 M H_2SO_4, the temperature was kept at 50° C and agitation at 150 rpm. All the parameters were monitored continuously in order to avoid non-enzymatic mechanisms. Hydrolysis was carried out for 3 h, which were the optimal autolysis conditions of anchovy protein (He et al. 2013b). The hydrolysis reaction was stopped by rising the temperature to 90° C for 15 minutes. The inactivated

homogenates were filtered through a filter press and centrifuged at 8000 rpm for 15 min at 4 °C. The supernatant was collected and dehydrated.

3.3. Powder preparation by spry dry technique

The drying of protein hydrolysates was carried out in a Mini Spray Dryer B-290 (Buchi Italia s.r.l) with a vanned wheel rotating at high speed and a concurrent drying chamber (0.8 m diameter and 0.6 m height). The solutions, stirred continuously at 30 °C, were fed into the chamber through a peristaltic pump at a constant flow rate (15 mL/min) and at inlet air temperature adjusted to 120 °C. Outlet air temperature, controlled by flow rate and inlet air temperature parameters, was adjusted to 65 °C. The dried powders were collected in a single cyclone air separator system.

3.4. Chemical analysis for powder characterization

3.4.1. ICP-MS analysis

Approximately aliquots of 0.5 g of powder were added to 1 mL internal Re standard at 0.8 μg/L, and then digested with 8 mL HNO_3 (65%, v/v) and 2 mL H_2O_2 (30%, v/v) in acid-prewashed PTFE vessels. The determinations of ^{9}Be, ^{24}Mg, ^{27}Al, ^{51}V, ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{75}As, ^{78}Se, ^{88}Sr, ^{107}Ag, ^{111}Cd, ^{118}Sn, ^{121}Sb, ^{208}Pb, ^{202}Hg in digested samples were carried out by7500CX Agilent ICP-MS spectrometer (ICPMS Agilent octapolo 7500 - Santa Clara, CA, USA) with a reaction/collision cell Octopolo Reaction System (ORS), and equipped with an automatic sample ASX 500. The sample was introduced with a flow of 1 mL min^{-1} and for each element three replicas have been set according to the procedures and operating conditions reported in Albergamo et al. (2017) and Pecoraro et al. (2017).

3.4.2. Protein determination

Protein content of powder was determined using the 938.08 AOAC methodology (AOAC, 1993).

3.4.3. NMR analyses

In order to carry out NMR analyses we have prepared two solutions namely called A and B. Solution A was made by D_2O solvent (suitable as a gross frequency and field homogeneity reference), by 10 mM of TSP (3-(Trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt) used as NMR frequency (δ =0.0 ppm) and quantification standard; and by 1 M PBS (phosphate buffer solution) with NaN_3 (0.2%) to inhibit bacterial growth. Solution B consisted of the water solution coming from the digestion treatment of the original samples. Samples were frozen at −40 °C until the analyses. All the B solutions were mixed with A solutions in a volumetric ratio 9:1. The resulting solution was poured in the 5 mm NMR test-tubes. These samples were all analysed at the same constant temperature (T = 298 K) with a NMR Bruker Avance III 500 MHz spectrometer equipped with a SMARTprobe with gradients. After the optimization of the field homogeneity, the 90° pulse duration to reach the maximum sensitivity (8.8 ± 0.1 μs; power attenuation 3Db), water presaturation power (25 Hz), acquisition time and time

delay, the quantification experiment is run according to the already assessed principles (Rotondo et al. 2019, Rotondo et al. 2017). The used pulse sequence was made by a pre-saturation pulse on the water signal followed by the noesy sequence (noesypresat). ^{1}H-NMR noesypresat experiments were performed with a spectral width of 12 ppm and 64 scans (8 dummy scans to reach the steady state before acquisition). As preliminary "inversion recovery" experiments sowed longitudinal relaxation times up to 4 seconds, the final noesypresat experiment included recycle delay over 20 seconds to guarantee the quantitative records. Free α-amino acids with several other metabolites are detected and quantified by NMR according to the MARA-NMR quantification technique and to the chemical shift assignment and characterization (Rotondo et al. 2014, Rotondo et al. 2015).

3.4.4. HPLC analyses

Amino acid composition was also assessed and confirmed by high performance liquid chromatography. Before the HPLC analysis the amino acids were subjected a to pre-column derivatization with 4-chloro-7-nitrobenzofurazan, 9-fluorenyl methylchloroformate, phenylisothiocyanate, naphthalene-2,3-dicarboxaldehyde, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, and o-phthaldialdehyde (OPA). The fluorescence of OPA derivatives was monitored at excitation and emission wavelengths of 340 and 455 nm, respectively. HPLC analysis was performed using the methods described in Zhaolai et al. (2014).

4. Conclusions

The paper describes the chemical amino acidic and elemental composition of protein hydrolysates obtained from *E. encrasicolu* waste. The obtained product displayed a remarkable presence of both EAA and other amino acids which are considered as key residues of oligopeptides assessed to relay beneficial effects in human health. This study enlighten great chances to recycle industrial by-products and to produce from these wastes new functional foods, taking advantage from new throughput technologies with low environmental impact and high economic sustainability. The outcomes concerning the nutritional value of the dry products are also supported by ongoing tests in animals possibly representing an intriguing continuation of this work. Eventually, such obtained products could be used as ingredient for functional food production, or also as stand-alone item.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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