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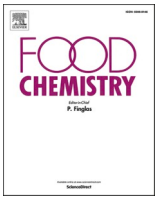

Production of small peptides and low molecular weight amino acids by subcritical water from fish meal: Effect of pressurization agent

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ABSTRACT

The hydrolysis of the water-soluble protein (WSP) fraction from tuna fish meal was evaluated by subcritical water (subW) by using N_2 and CO_2 as different pressurization agents in the temperature range from 140 to 180 °C. For both gases, the amino group release increased by increasing working temperature while the Lowry response decreased due to production of smaller-size peptides and free amino acids.

The free amino acid content was higher with CO_2 than with N_2. At 180 °C, 344 ± 5 and 275 ± 3 mg of free amino acids per g of WSP were released, respectively; although, in both systems the smallest molecular weight amino acids, glycine and alanine, were preferentially released. The free amino acids content obtained by enzymatic hydrolysis with commercial proteases Alcalase and Novozym was much lower with the highest hydrolysis yield determined for histidine. These results have been supported by size exclusion chromatography analysis.

1. Introduction

Fish meal is an essential ingredient in aquaculture and pet-food industry since it presents a high protein content as well as a valuable lipid fraction composition. However, new sources of protein, such as those derived from fermentation processes, plant-based or insect protein, force fish meal producers to look for new valuable products in the fishmeal production chain.

Protein from marine origin is of high nutritional value and presents a good profile of essential amino acids (Guérard et al., 2001). Exploring the production of small peptides and valuable free amino acids from marine protein, and specifically from fish meal protein, is of great interest according to their well-documented functional properties and other interesting bioactivities. New functional and healthy products can be obtained creating more sustainable and environmentally friendly processes (Petrova et al., 2018; Slizyte et al., 2016).

Hydrolysis converts protein into small peptides of great interest in the food, pharmaceutical and cosmetic industries. Chemical and enzymatic hydrolysis are widely employed in the hydrolysis process of proteins. However, enzymes are expensive and time consuming, while chemical hydrolysis usually requires the use of concentrated acids or alkalis and generates a high salt content in the final product after neutralization (Ahmed and Chun, 2018).

Large scale production of peptides will require the development of new alternative cost-effective approaches to meet the quality and functionality specifications demanded by the market (Melgosa et al., 2020). In this regard, the use of subcritical water as hydrolytic agent offers a green and modern alternative to the traditional methods.

Subcritical water (subW) is water in its liquid state in the range from 100 to 374 °C and pressures up to 22 MPa. Under these conditions, water presents unique properties, the dielectric constant decreases facilitating the solubilization of apolar components. Furthermore, the viscosity and density decrease. Under subcritical water conditions there is also an increase of the ionic product. The increment in the concentration of H^+ and OH^- in the aqueous medium raises its activity as an acid- or base-like catalyst for hydrolysis reactions (Marcet et al., 2016). Based on these properties, the mechanism of hydrolysis of peptide bonds in the protein structure in the presence of subW is due to the acid-catalysis effect due to the increase of the ionic product (Lamp et al., 2020). subW has been successfully applied to hydrolyze and fractionate the protein content of different herbaceous and aquatic biomass (Rivas-Vela, Amaya-Llano, Castaño-Tostado, & Castillo-Herrera, 2021; Trigueros et al., 2021a) as well as from crops (Alonso-Riaño et al., 2021; Sganzerla et al., 2022). subW treatment may be a promising alternative to produce different bio-

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compounds from different biomasses; however, there appears to be a lack of data for subcritical biomass process reactor systems, in particular for the production and recovery of proteins and amino acids at a larger scale (Di Domenico Ziero et al., 2020). Regarding scaling up process, temperature control becomes primordial, since the increasing temperature rate for different capacity reactor systems can play an effect on the hydrolysis yield of some of the biopolymers (Alonso-Riaño et al., 2023; Di Domenico Ziero et al., 2020). Therefore, temperature is one of the most critical parameters in subW treatment.

During processing of fish and fish canning, considerable amount of fish-derived water-soluble proteins are discarded into the waste water (Iwata et al., 2000). Therefore, a two-stage process for fish meal valorisation is proposed based first on the valorisation of the water-soluble protein (WSP) fraction and subsequently the non-water-soluble protein, in a cascade approach, contributing to increase the value of the marine resources. The easy fractionation of the protein from the fish meal will offer an economical route for amino acid production. The WSP is no longer bound to the matrix of the raw material and it is expected a high yield of free amino acid release compared with the total protein fraction treatment.

There are only a few works dealing with the choice of the gas to generate the working pressure in subW treatment. However, it has been proved to be a factor to consider (Di Domenico Ziero et al., 2020). According to the literature, the hydrolysis of biopolymers in subW can be significantly enhanced by adding carbon dioxide to the medium since it leads to a more acidic medium due to the formation of carbonic acid that serves as a catalyst according to (Rogalinski et al., 2008):

$$CO_2 + H_2O \rightarrow H_2CO_3 \rightarrow H^+ + HCO_3^- \rightarrow 2\,H^+ + CO_3^2 \quad (1)$$

The effect of carbon dioxide on the subW hydrolysis of different biopolymers has been reported in the literature. Rogalinski et al. (2008) reported that the addition of carbon dioxide led to an increase in amino acid production from bovine serum albumin (BSA) compared to pure subW. With other polymers such as xylan, van Walsum (2001) showed that carbonic acid substantially increased hydrolysis activity in comparison to water alone. However, for aspen wood and corn stoves the addition of carbonic acid during subW treatment did not bring any further improvement in the subsequent enzymatic hydrolysis step. This was attributed to the autocatalytic effect of acetyl groups released from the raw materials during hydrolysis, which performed a similar role to that of carbonic acid and obscured its action when added to the reaction media (Yourchisin and Van Walsum, 2004).

The objective of this work was to investigate the hydrolysis of the water soluble protein fraction of the fish meal by subW regarding the production of peptides and free amino acids. Hydrolysis kinetics were determined at different temperatures and with different pressurization agents, including an inert gas, such as nitrogen and carbon dioxide as an improvement agent for protein hydrolysis. Hydrolysis driven by subW was compared with enzymatic hydrolysis by commercial proteases considering the amount and profile of free amino acids released and antioxidant capacity. The different results obtained for subW and enzymatic hydrolysis were correlated with the molecular weight distribution of the hydrolysates.This treatment could lead to a feasible solution for a first step for fish meal valorisation via previous fraction and hydrolysis of the WSP.

2. Experimental section

2.1. Raw material

The raw material used in this work was fish meal from tuna (*Thunnus* sp.) kindly supplied by Sarval Bio-Industries Noroeste, S.A.U. (A Coruña, Spain). The raw material was kept at 4 °C until used. Moisture content was 3.4 % ± 0.1 % (w/w) as determined gravimetrically after drying preweighted samples at 105 °C for 24 h.

Ash content was determined after subjecting the sample at 500 °C for 12 h. Lipid content of the fish meal was determined by Soxhlet extraction (Buchi B-8111) using hexane as solvent. Protein content of the raw material was determined from the nitrogen elemental content applying the corresponding *N*-factor obtained by the amino acid profile of the fish meal protein.

2.2. Water soluble protein extraction

Water soluble protein, WSP, was extracted by putting in contact water with the fish meal in a thermostated extractor. Extraction was carried out at different fish meal:water ratios (4:100, 8:100, 16:100 and 24:100) and different temperatures (20, 50 and 80 °C). Protein extraction kinetic was followed along time by withdrawing samples at regular time intervals. Samples were stored at −18 °C until analysis. The quantification of the protein was carried out by the Lowry method. The yield of WSP was evaluated according to:

$$Yield\ of\ WSP = m_{WSP}/m_{totalprotein} \quad [1]$$

where m_{WSP} is the amount of WSP in the supernatant solution after extraction with water and $m_{total\ protein}$ is the total amount of protein in the fish meal.

2.3. Equipment for subcritical water hydrolysis

Subcritical water hydrolysis of WSP fraction was carried out in a lab-assambled batch system with a reactor of 0.5 L capacity. The reactor was covered by a ceramic heating jacket (230 V, 4000 W, ø 95 mm, 160 mm height) to reach the selected working temperature. A Pt100 sensor placed inside the reactor and the PID system to which it is connected allowed to control and register the temperature during the extraction. A needle valve (Autoclave Engineers) followed by a cooling system was connected to collect samples along the hydrolysis.

In a typical run, 200 mL of the WSP extract was charged into the reactor. Three different temperatures were essayed, 140, 160 and 180 °C and the working pressure was set at 50 bar by using different gases. This pressure was chosen based on previous studies of the research group for biomass valorization from different sources (Alonso-Riaño, Ramos, Trigueros, Beltrán, & Sanz, 2023; Trigueros et al., 2021b). The use of mild reaction pressure, 40 to 60 bar, has been found also as optimum for the hydrolysis of proteins of biomass waste (fish) to produce free amino acids (Cheng et al., 2008). Furthermore, according to literature (Rivas-Vela et al., 2021) the effect of pressure on performance of hydrolysis in different studies has been observed as a non-significant, compared with temperature and time, as long as water remains in the liquid state.

Pressurization of the vessel was performed with inert N_2 or CO_2, fed through a sintered stainless steel micro-filter with a pore size of 10 μm submerged in the liquid sample (Illera et al., 2018). The same amount of CO_2 (8.3 g) was added to all the experiments by using a syringe pump with pressure and volume controllers (ISCO 260 D), yielding a ratio of 2 g CO_2/g WSP). Once the pressure was reached, there was no need of further gas addition into the high pressure reactor.

Hydrolysis kinetics were followed by carefully withdrawing samples at regular time intervals through the sampling port. After 300 min, the vessel was cooled and it was depressurized when the temperature was lower than 90 °C.

2.4. Enzymatic hydrolysis

Two different commercial proteases, kindly donated by Novo Industry, were selected in this work, specifically Alcalase® and Novozym®. Firstly, the protease activity of the two commercial enzymes was determined by the casein method according to Pokhum et al. (2015) with some modifications. One mL of casein solution (2% w/v) prepared in potassium phosphate buffer (50 mM) was incubated in a water bath at

37 °C for 5 min. Then, 500 μL of the commercial protease enzyme was added and incubated at 37 °C for 10 min. The reaction was stopped by adding 3 mL of 0.4 M trichloroacetic acid. After that, the mixture was filtrated through a 0.45 μm filter. One mL of the filtrated mixture was mixed with 1 mL of the Folin Cioucalteau's reagent and 5 mL of 0.50 M Na_2CO_3. The mixture was incubated at 37 °C for 30 min. The protease digests casein and the amino acid tyrosine is liberated, along with other amino acids and peptide fragments, that react with the Folin's reagent producing a blue-colored chromophore.

Protease activity was determined by measuring the absorbance at 660 nm in a Jasco (V-750) spectrophotometer. The blank sample was prepared by adding the stopping reagent to the mixture before the sample of enzyme. A calibration curve was prepared by reacting known quantities of tyrosine with the Folin's reagent and protease activity was expressed as μmol of tyrosine released per mL of enzyme, resulting 1157 ± 74 and 826 ± 33 μmol of tyrosine/mL for Alcalase® and Novozym®, respectively.

In the enzymatic hydrolysis study, 100 mL of the WSP extract obtained was incubated at 60 °C and pH of 8 by adding 225 U of each protease. Samples were withdrawn at regular time intervals and subsequently heated in boiling water for 5–10 min to inactivate the protease. Samples were frozen at −18 °C until analysis.

2.5. Analytical methods

2.5.1. Lowry method for protein determination

The Lowry method was carried out to determine the WSP fraction during its extraction, as well as during the subW and enzymatic hydrolysis treatments (Lowry et al., 1951). Briefly, the samples were conveniently diluted to 1 mL with deionized water. 5 mL of Lowry reagent were added (Lowry reagent: 20 $g{\cdot}L^{-1}$ sodium potassium tartrate, 20 $g{\cdot}L^{-1}$ copper sulfate pentahydrate and 20 $g{\cdot}L^{-1}$ sodium carbonate in 0.1 M NaOH; in proportion 1:1:100). Samples were incubated for 15 min in the dark. Afterwards, 0.5 mL of Folin-Ciocalteu's phenol reagent, diluted 1:3 in distilled water, were added, and the mixture was let to stand for 30 min in the dark. Finally, absorbance was measured at 750 nm using a Jasco spectrophotometer and protein content was obtained based on a calibration curve using bovine serum albumin as standard.

2.5.2. Ninhydrin assay

The ninhydrin reaction method was carried out according to the Sigma Aldrich protocol. Ninhydrin assay results in the formation of soluble chromophores by all primary amines, including amines, amino acids, proteins and even ammonia (Friedman, 2004). Briefly, 2 mL of the diluted protein sample were gently mixed with 1 mL of ninhydrin reagent solution and placed into a boiling water bath for 10 min. After cooling, 5 mL of 95% ethanol were added. Ninhydrin reagent solution was purchased from Sigma-Aldrich and leucine was used as standard (Friedman, 2004). Absorbance was measured at 570 nm.

2.5.3. Amino acid profile

The amino acid profile was analyzed by gas chromatography after derivatization by using the EZ:faast^{TM} kit (Phenomenex) and a GC-FID instrument (Hewlett Packard, HP 2890 Series). Details can be found elsewhere (Alonso-Riaño, Sanz, Benito-Román, Beltrán, & Trigueros, 2021; Trigueros et al., 2021a). Amino acid profile of fish meal and WSP fraction was determined after hydrolysis using 6 N HCl at 100 °C during 24 h. Tryptophan and cysteine are lost by acid hydrolysis, and methionine can be partially destroyed by acid hydrolysis, so an alkaline hydrolysis was also carried out to determine these amino acids (Alonso-Riaño et al., 2021). Arginine can not be detected by this kit and asparagine and glutamine were quantified as aspartic and glutamic acids, respectively.

For WSP extracts, amino acids were determined before the HCl hydrolysis and referred to as free amino acids. After hydrolysis with HCl of the WSP, total amino acids were determined and the diference between the value of amino acids obtained after HCl hydrolysis and free amino acids were referred to as constituted amino acids of the WSP. Free amino acid were also determined during enzymatic and subW hydrolysis.

2.5.4. Elemental analysis

Elemental composition (C, H, N, S) was determined by an organic elemental micro-analyzer equipment (Thermo Scientific Model Flash 2000). Nitrogen composition of the sample and its amino acid profile were used to calculate the N factor of the fish meal protein.

2.5.5. Determination of antioxidant capacity

The antioxidant activity of the WSP and its hydrolysates was assesed by the Ferric Reducing Antioxidan Power (FRAP) assay according to Benzie and Strain (1996). As standard, a solution of $FeSO_4{\cdot}7H_2O$ (0.1 M) was used. Results were expressed in μmoles of Fe^{2+} per gram of dry WSP.

2.5.6. Size exclusion chromatography

The molecular weights (MW) of the WSP and its hydrolysates were determined employing high-pressure size exclusion chromatography coupled to a refraction index detector (HPSEC-RID, 1260 HPLC system, Agilent Technologies, CA, USA). Samples and standards were filtered through 0.45 μm syringe filters. A PL Aquagel guard column was linked in series with PL Aquagel-OH 30 (from 0.1 to 60 kDa) and PL Aquagel-OH 40 (from 10 to 200 kDa) columns from Agilent Technologies (300 mm × 7.5 mm, particle size 8 μm). Characterization of WSP and its hydrolysates was performed in isocratic mode with 0.01 M NH_4Ac, at a flow rate of 0.7 mL/min at 40 °C. A PEO/PEG standard set (117.9 – 0.194 kDa) was used for calibration and data were analyzed with Agilent OpenLab Data Analysis 2.5 software.

2.6. Statistical analysis

All values were expressed as mean ± standard deviation of at least two replicates. The significance differences between the mean value from the different factors was determined based on an analysis of the variance with the Fisher's Least Significant Difference (LSD) method at p-value ≤ 0.05. Correlation between the reducing capacity of the hydrolysates and the amino group released and Lowry assay results was determined by using the Pearson's Correlation Test. The software Statgraphics19 X64 was used.

3. Results and discussion

3.1. Characterization of the fish meal

Results were presented in a dry basis taking into account the moisture content of the fish meal. Ash content of fish meal was 21.7 ± 0.2 % (w/w). Total lipid content was 6.5 ± 0.2 % (w/w) as determined by Soxlhet extraction. The elemental analysis composition was: 40 ± 1 % (w/w) of C, 10.3 ± 0.3 % (w/w) of N, 5.8 ± 0.1 % (w/w) of H and 0.45 ± 0.07 % of S (w/w). Crude protein was obtained from the nitrogen content and the corresponding conversion factor to estimate the crude protein content. The Nitrogen factor was calculated from the amino acid profile of the fish meal (see Table 1) according to the NREL standard protocols (Hames et al., 2008). For the fish meal used in this work, a *N*-factor of 5.0 was determined. This value was in the same range as the one reported by Salo-Väänänen and Koivistoinen (1996) for fish and fish products with a *N*-factor of 4.94. This value indicated that a significant amount of nitrogen comes from other non-amino acids structures, such as those compounds derived from degradation of proteins or other non-protein nitrogen compounds. The *N*-factor values of 5.0 yielded a crude protein content of 51 ± 2 % (w/w). However, in literature many authors still consider 6.25 as the *N*-factor to convert nitrogen into crude protein values for fish and fish products (Ween et al., 2017). A *N*-factor of 6.25 will yield a crude protein of 64 % (w/w), which is closer to the value reported by the provider.

Table 1
Amino acid profile of the raw fish meal product and of the water soluble protein (WSP). Free amino acid profile of subW and enzymatic hydrolysates.

Amino acid	Fish meal, mg aa/g_{prot}	WSP, mg aa/g WSP		Free amino acids generated during hydrolysis mg aa/g WSP							
				subW hydrolysis in N_2			subW hydrolysis in CO_2			Alcalase	Novozym
		Free aa	Bound aa	140 °C	160 °C	180 °C	140 °C	160 °C	180 °C	60 °C	60 °C
ALA	89 ± 3	8.2 ± 0.1	80 ± 7	18.8 ± 0.1^{b}	52.6 ± 0.1^{d}	66.0 ± 0.4^{e}	19.8 ± 0.4^{c}	66.3 ± 0.2^{f}	72.8 ± 0.4 ^{g}	9.7 ± 0.1^{a}	9.8 ± 0.1^{a}
GLY	86 ± 2	3.5 ± 0.1	195 ± 9	30.3 ± 0.2^{c}	95 ± 2^{d}	110 ± 1^{e}	27 ± 0.1^{b}	118.4 ± 0.3^{f}	130.4 ± 0.3 ^{g}	5.4 ± 0.1^{a}	4.3 ± 0.3^{a}
VAL	43 ± 3	4.0 ± 0.1	25 ± 5	4.5 ± 0.2^{a}	7.9 ± 0.9^{c}	10.4 ± 0.1^{d}	6.7 ± 0.1^{b}	8.0 ± 0.1^{c}	13.3 ± 0.1^{e}	5.03 ± 0.07^{a}	4.7 ± 02^{a}
LEU	63 ± 4	5.0 ± 0.1	31 ± 2	5.1 ± 0.3^{a}	8.5 ± 0.2^{d}	11.0 ± 0.1^{e}	6.8 ± 0.1^{c}	11.0 ± 0.1^{e}	13.4 ± 0.1^{f}	5.9 ± 0.1^{b}	5.7 ± 0.1^{b}
ILE	36 ± 2	2.7 ± 0.1	15 ± 2	2.2 ± 0.1^{a}	2.1 ± 0.2^{a}	3.4 ± 0.1^{b,c}	3.5 ± 0.1^{c}	2.2 ± 0.1^{a}	3.8 ± 0.1^{b,c}	3.4 ± 0.1^{c}	3.2 ± 0.1^{b}
THR	43 ± 2	2.4 ± 0.1	29 ± 2	1.7 ± 0.4^{c}	0.7 ± 0.2^{a,b}	0.43 ± 0.03^{a}	2.4 ± 0.1^{d}	0.59 ± 0.05^{a,b}	5.0 ± 0.1^{b,c}	2.5 ± 0.4^{d}	2.7 ± 0.1^{d}
SER	44 ± 3	1.6 ± 0.1	34 ± 5	6.3 ± 0.1 ^{g}	0.42 ± 0.05^{a}	0.43 ± 0.02^{a}	6.0 ± 0.1^{f}	1.1 ± 0.2^{c}	0.8 ± 0.1^{b}	2.44 ± 0.04^{e}	2.0 ± 0.2^{d}
PRO	58 ± 3	3.2 ± 0.1	108 ± 5	8.3 ± 0.1^{c}	25.7 ± 0.8^{d}	41.9 ± 0.6^{f}	8.4 ± 0.1^{c}	27.9 ± 0.1^{e}	52 ± 1 ^{g}	3.6 ± 0.1^{b}	3.2 ± 0.1^{a}
ASP	94 ± 2	2.8 ± 0.1	67 ± 7	7.8 ± 0.7^{b}	2.2 ± 0.8^{a}	2.0 ± 0.1^{a}	11.4 ± 0.1^{c}	3.0 ± 0.1^{a}	2.4 ± 0.1^{a}	3.0 ± 0.1^{a}	2.8 ± 0.1^{a}
MET	27 ± 3	1.6 ± 0.1	11 ± 2	1.8 ± 0.1^{a}	2.7 ± 0.4^{c}	2.2 ± 0.1^{b}	2.5 ± 0.1^{c}	5.7 ± 0.2^{d}	6.0 ± 0.3^{d}	2.00 ± 0.03^{a,b}	2.0 ± 0.1^{a,b}
HYP	24 ± 3	n.d.	58 ± 9	3.0 ± 0.1^{b}	7 ± 1^{b}	6.9 ± 0.1^{b}	2.3 ± 0.1^{b}	7.8 ± 0.7^{b}	12 ± 1^{c}	0.36 ± 0.01^{a}	0.24 ± 0.03^{a}
GLU	110 ± 6	2.5 ± 0.1	100 ± 14	0.7 ± 0.1^{a,b}	0.8 ± 0.1^{b}	4.8 ± 0.03^{e}	2.0 ± 0.1^{c}	0.63 ± 0.03^{a}	9.2 ± 0.2^{f}	1.93 ± 0.02^{c}	3.7 ± 0.1^{d}
PHE	34 ± 2	2.5 ± 0.1	18 ± 2	2.6 ± 0.1^{a}	4.0 ± 0.1^{c}	5.3 ± 0.1^{d}	3.1 ± 0.1^{b}	5.2 ± 0.1^{d}	6.3 ± 0.1^{e}	3.1 ± 0.1^{b}	3.0 ± 0.1^{b}
LYS	57 ± 4	2.4 ± 0.1	35 ± 6	3.5 ± 0.1^{a}	6 ± 1^{b}	3.7 ± 0.1^{a}	3.8 ± 0.1^{a}	6.8 ± 0.2^{c}	6.2 ± 0.2^{b,c}	3.5 ± 0.1^{a}	4.0 ± 0.1^{a}
HIS	22 ± 2	13.3 ± 0.1	13 ± 2	7.9 ± 0.4^{c}	5 ± 2^{b}	2.8 ± 0.1^{a}	12.6 ± 0.4^{d}	6.9 ± 0.2^{b,c}	6.9 ± 0.1^{b,c}	19.0 ± 0.7^{f}	16.1 ± 0.2^{e}
HLY	1.9 ± 0.4	0.5 ± 0.1	52 ± 18	0.5 ± 0.1^{c}	0.40 ± 0.05^{a,b}	0.43 ± 0.03^{b,c}	1.0 ± 0.1^{e}	0.8 ± 0.1^{d}	n.d.	0.25 ± 0.02^{a}	0.39 ± 0.04^{a,b}
TYR	26 ± 2	1.9 ± 0.1	13 ± 3	2.1 ± 0.1^{a}	2.2 ± 0.3^{a,b}	2.5 ± 0.1^{b,c}	2.6 ± 0.1^{c}	2.7 ± 0.1^{c,d}	2.9 ± 0.1^{d}	2.7 ± 0.1^{c,d}	2.5 ± 0.1^{b,c}
TRP	8 ± 1	0.5 ± 0.1	53 ± 12	0.26 ± 0.02^{a}	0.27 ± 0.03^{a}	0.94 ± 0.02^{e}	0.40 ± 0.04^{b}	0.27 ± 0.03^{a}	0.81 ± 0.05^{b}	0.72 ± 0.03^{d}	0.66 ± 0.04^{c}
CYS	2.3 ± 0.6	0.3 ± 0.1	25 ± 9	0.2 ± 0.1 ^{a,b,c}	0.28 ± 0.06^{b,c}	0.16 ± 0.02^{a,b}	0.30 ± 0.06^{b,c}	0.27 ± 0.06^{c}	0.26 ± 0.03^{b,c}	0.11 ± 0.01^{a}	0.26 ± 0.09^{b,c}
TAA	868 ± 48	59 ± 2	962 ± 121	108 ± 4^{b}	224 ± 10^{d}	275 ± 3^{e}	123 ± 2^{c}	276 ± 3^{e}	344 ± 5^{f}	75 ± 1^{a}	71.0 ± 0.6^{a}
EAA	333 ± 23	34.3 ± 0.3	230 ± 35	30 ± 2^{a}	37 ± 5^{b}	40.1 ± 0.7^{c}	31.9 ± 0.7^{c,d}	42.4 ± 0.9^{e}	51.4 ± 0.2^{f}	45 ± 1^{e}	42.0 ± 0.2^{d}
Yield %				11 ± 1^{b}	22 ± 2^{d}	27 ± 3^{e}	12 ± 1^{c}	27 ± 3^{e}	34 ± 3^{f}	7.3 ± 0.9^{a}	7.0 ± 0.9^{a}

TAA: total amino acids, EAA: total essential amino acids, n.d.: non detected. Values with different letters in each raw are significantly different when applying the LSD method at p-value ≤ 0.05. ALA: alanine, GLY: glycine, VAL: valine, LEU: leucine, ILE:isoleucine, THR: threonine, SER:serine, PRO: proline, Asp: aspartic acid, MET: methionine, HYP: hydroxyproline, GLU: glutamic acid, PHE: phenylalanine, LYS: lysine, HIS: histidine, HLY: hydroxylysine, TYR: tirosine, TRP: triptophan, CYS: cysteine.

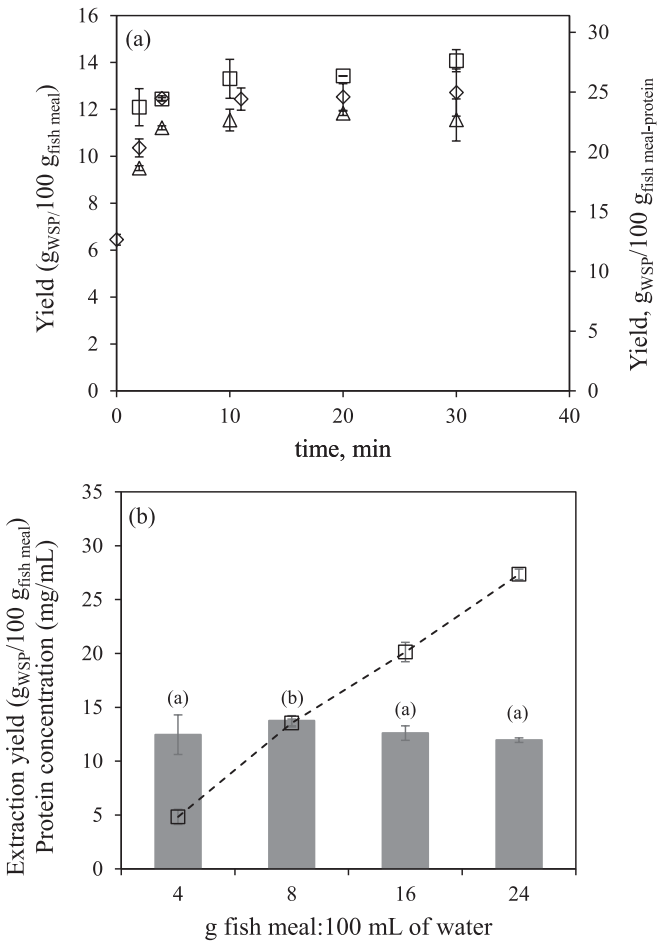

Fig. 1. (a) WSP extraction kinetics at different temperatures (△ 20 °C, ◇50 °C, □ 80 °C) and a fixed ratio of 8 g of fish meal: 100 g of water (b) Effect of the solid to solvent ratio on the WSP extraction yield (g_{WSP}/100 g fish meal) and protein concentration (mg/mL, □). Different letters in the extraction yield indicate means significantly different by using the LSD procedure at p-value ≤ 0.05.

3.2. Extraction and characterization of the water soluble protein fraction of fish meal

WSP was extracted at different temperatures (from 20 to 80 °C) at a fish meal to solvent ratio of 8 g fish meal:100 mL of distilled water. Total protein content in the liquid supernatant was determined according to the Lowry assay. Extraction kinetics curves are presented in Fig. 1a. Faster initial extraction rates were obtained by increasing temperature. Additionally, final WSP yield was also slightly higher at the highest temperature essayed in this work yielding values of 14.1 ± 0.5 g of WSP per 100 g of dry fish meal, which represented 28 % of the total protein content in the fish meal.

The effect of fish meal to solvent ratio (w/v) on the WSP extraction was studied at 80 °C in the range from 4 to 24 g of fish meal per 100 mL of distilled water. Results at the final extraction time of 30 min are presented in Fig. 1b. Fig. 1b shows that the extraction yield, WSP/100 g fish meal, remained more or less constant in the range considered in this work, while the WSP concentration in the extract (mg WSP/mL) increased as the fish meal to solvent ratio increased. Since the extraction yield was of the same order, the WSP fraction was therefore more concentrated by decreasing the amount of solvent. However, the WSP extract obtained at the ratio 16 g fish meal:100 mL of distilled water was used for further hydrolysis studies instead of the highest fish meal:solvent ratio essayed (24:100) due to poor separation of the non-water soluble solid after extraction at this condition, since small particles and suspended solids were perceptible by the human eye after centrifugation of the extracts. The ratio of total soluble solids (SS) per g of dry fish meal was around 0.25 g SS /$g_{dry\ fish\ meal}$ for any of the fish meal to solvent ratios studied. Therefore, according to the Lowry assay, around half of the soluble solids correspond to solids of protein nature.

Characterization of the WSP was done in terms of the amino acid profile. Free and bound amino acids of the WSP have been listed in Table 1. The percentage of free amino acids regarding the total content of amino acids in the WSP extract was 5.8 % (w/w). Similar to the original fish meal, the major amino acids in the WSP extract were alanine, glycine, proline and glutamic acid; however, the content of glycine and proline was nearly double in the WSP extract than in the original fish meal protein.

3.3. Hydrolysis of water soluble protein by subcritical water.

The WSP extract was subjected to hydrolysis by subW at different temperatures from 140 to 180 °C. Pressure was fixed at 50 bar by using two different gases as presurizaton agents, N_2 and CO_2.

3.3.1. Release of amino group

To follow the hydrolysis of the protein fraction, samples were withdrawn during the experiment and the ninhydrin assay was performed to determine the total number of primary amines released to the medium. Firstly, the initial content of primary amines in the WSP extract was determined, resulting 34 ± 1 meq leucine/L (1.7 ± 0.2 meq leucine/g WSP). However, the number of free amino acids determined in the characterization of the WSP extract (see Table 1) was much lower as determined by the sum of individual free amino acids determined by GC (59 ± 2 mg/g WSP or 0.475 ± 0.004 mmols/g WSP or 9.4 ± 0.1 mM). The difference could be attributed to the compounds determined by the ninhydrin test, since the test would include all type of primary amines such as peptides, proteins, amino acids, amines and even ammonia (Friedman, 2004). As it has been been widely reported in the literature, during processing of fish products, degradation of proteins can take place, leading to the formation of different non-protein nitrogen compounds such as biogenic amines, total volatile basic nitrogen, trimethylamine, dimethylamine and ammonia (Folador et al., 2006). Some of these compounds can yield the characteristic chromophore of ninhydrin with primary amino groups.

Fig. 2a and 2b show the kinetics for the release of amino groups in subW, as determined by the ninhydrin assay, at the three different temperatures studied, 140, 160 and 180 °C, by using nitrogen or CO_2 as pressurizing agents, respectively. During protein hydrolysis, peptide bonds were broken, which resulted in an increase of primary amine concentration corresponding to an increase in the degree of hydrolysis of WSP.

An increase in temperature led to a faster and higher release of amino groups due to the hydrolytic action of subW for both pressurization gases. The initial rate of amino groups released was evaluated at the three temperatures, determining statistically significant differences in the initial slopes considering the temperature as the factor at the 99 % confidence level for both pressurization agents (Table S1). Similar effect of temperature on protein hydrolysis degree has been previously reported for different types of biomass and model proteins such as bovine serum albumin (Alonso-Riaño, Sanz, Benito-Román, Beltrán, & Trigueros, 2021; Koh, Lee, Ramachandraiah, & Hong, 2019; Trigueros et al., 2021b) where an increase in temperature led to an increase in the release of amino group under subW conditions. In the temperature range covered in this work, the ionic product of water, K_w, increases with temperature due to the high levels of hydronium (H_3O^+) and hydroxide (OH^-) ions that favour protein hydrolysis.

Regarding the effect of the addition of CO_2 as pressurization agent instead of N_2, slightly higher values of the initial release of amino groups were obtained by using CO_2 at the different temperatures essayed,

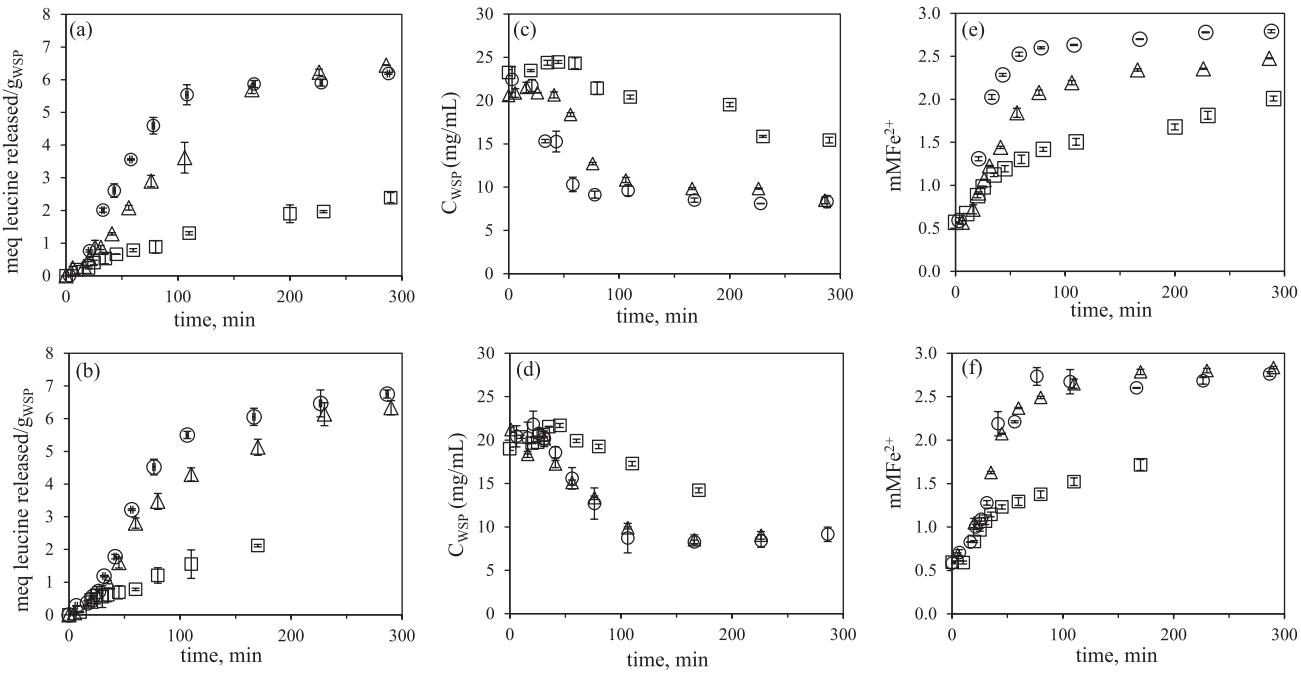

Fig. 2. (a,b) Amino groups release kinetics by subW; (c,d) Evolution of the total protein content in subW hydrolysates; (e,f) Evolution of the reduction capacity of subW hydrolysates with different pressurization agents (a,c and e) N_2 (b, d and f) CO_2 at different temperatures (□ 140 °C, △ 160 °C, ○180 °C).

except for 180 °C; however, the final value of amino group release was similar for both systems. Therefore, the expected enhacement of the acidic behavior of the medium due to the addition of CO_2 was hardly observed by the ninhydrin method. It could be concluded that the ninhydrin assay allowed us to qualitatively follow the protein hydrolysis, but the presence of different primary amines in the medium could interfere in the determination of free amino acid release.

3.3.2. *Total protein content in the subW hydrolysates of soluble water protein*

Total protein content was monitored by the Lowry assay along subW treatment. It was observed a decrease in the total protein content being faster by increasing the operating temperature for both pressurization agents (N_2 and CO_2, Fig. 2c and 2d, respectively).

The hydrolysis of the WSP into small peptides and free amino acids might be responsible for the decrease response in the Lowry assay. According to the literature, with the exception of tyrosine and tryptophan, free amino acids will not produce a colored product with the Lowry reagent, although most dipeptides can be detected (Schaich, 2016). Furthermore, for peptides, color formation increases with peptide size as well as with the presence of tyrosine, tryptophan, cysteine, histidine and asparagine in the peptide or protein backbone. This fact has been also observed in this work, since a mixture of pure amino acids standards (in a similar concentration as the one obtained in the hydrolysis of WSP by subW at 180 °C in the presence of CO_2, see section 3.3.3) did not produce an appreciable colored product. Additionally, solutions of protein standards of different molecular weight (see Fig. S1) were prepared at the same concentration (0.2 mg/mL) and the Lowry response, as absorbance, was determined. Fig. S1 shows that proteins above 40–50 kDa yielded a similar response to the Lowry assay. However, for smaller proteins and peptides the response is generally lower than for molecules of higher molecular weight and it seems to be affected by the amino acid composition. Similar conclusions were reached by Rodrigues et al. (2021) in the protein hydrolysates from shellfish waste streams obtained by using subW extraction.

To assure that no protein or peptides degradation was taking place at the working conditions essayed in this work, total nitrogen was determined by TOC analysis during the subW treatment carried out at 180 °C in the presence of CO_2. Assuming the same *N*-factor as for total WSP, the total protein fraction in the subW extracts remained more or less constant with values of 22 ± 2 mg/mL. Therefore, it can be concluded that when the Lowry method is used to quantify the total solubilized protein in different hydrolysis studies, small peptides obtained could led to a lower response, as it has been demonstrated in this work.

3.3.3. *Free amino acid profile in the subW hydrolysates*

Determination of amino group release by the ninhydrin assay could overestimate the WSP hydrolysis yield into free amino acids and small peptides, since the test would include other primary amines (Friedman, 2004). Therefore, it is important to quantify the total undamaged free amino acids formed in the hydrolysate.

Individual free amino acids content expressed as mg aa/g WSP is listed in Table 1 for subW treatment. It can be clearly observed that total free amino acid content, evaluated as the sum of the individual free amino acid determined by GC, increased by increasing temperature due to an increase of the dissociation constant of water creating a more acidic medium. Other works have found a maximum in the total free amino acid content in subW hydrolysates from different protein sources at much higher temperature than the maximum temperature studied in this work. For instance, Quitain et al (2001) determined a maximum at 523 K, 4 MPa and 60 min of reaction time in a batch configuration in the subW hydrolysis of the protein from shrimp shells (around 233 mg of free amino acids per g of shell protein).

Althgough the maximum temperature would be different for each amino acid, for the predominant amino acids determined in the WSP hydrolysate, no degradation was taking place at the working conditions of this work (maximum temperature of 180 °C and 288 min treatment time). A common factor used when working with subW is the severity factor that combines the effect of treatment time and temperature according to the following expression (Alonso-Riaño et al., 2021):

$$\log R_o = \log\left(t \cdot \exp\left(\frac{(T - T_{ref})}{14.75}\right)\right) \quad [2]$$

where t is the treatment time (min), T is the operating temperature (°C) and T_{ref} is equal to 100 °C. In the literature, a maximum in the free

amino acids production was obtained at severity factors around 5.9–6.2 from different protein sources (Quitain et al., 2001; Rogalinski et al., 2005); while the highest severity factor applied in this work corresponds to 4.8 (at 180 °C and 288 min treatment time). Therefore, according to the maximum observed in the literature for free amino acids at higher severity factors no degradation was observed in this work, at least for the predominant free amino acids formed in the subW hydrolysate.

The amount of free amino acids in subW hydrolysates was significantly higher in the presence of CO_2 compared with N_2, at all the working temperatures essayed in this work. For instance, by working at 180 °C, 275 ± 3 and 344 ± 5 mg free aa/g WSP were obtained in N_2 and CO_2, respectively (25 % higher in CO_2 than in N_2). According to Rogalinski et al. (2005) the solubility of CO_2 in water increases significantly in the subcritical region, increasing the concentration of protons in the medium enhacing the formation of free amino acids. These authors determined that the addition of CO_2 in the hydrolysis of bovine serum albumin as model protein induced the formation of 150.3 mg aa/g_{BSA}, obtaining 36.6 mg aa/g_{BSA} in the absence of CO_2. In the present work, the addition of CO_2 did not lead to such big increase, but around 14–25 % of increase was observed. These values were of the same order as the one reported by Zhu et al. (2010) who determined that the addition of CO_2 had also an effect on the amino acid production from bean dregs, with values around 20 % higher by using CO_2 instead of air. However, other works found different trends for each individual amino acid by using N_2, CO_2 and air as pressurization agents in the hydrolysis of fish meat by subW (Zhu et al., 2008).

In any case, in either of the two pressurization agents, N_2 or CO_2, free amino acids were produced with a similar qualitative profile. As it can be observed in Table 1, the major amino acids in the different hydrolysates were mainly alanine and glycine, followed by proline. These three amino acids accounted for 81 % and 74 % of the total free amino acids determined in the subW hydrolysates in N_2 and CO_2, respectively. Alanine and glycine were also determined as the most abundant amino acids in the subW hydrolysates of other protein sources such as hog hair (Esteban et al., 2010), entrails of fish meat (Kang et al., 2001), shrimp shells (Quitain et al., 2001) and pure protein such as bovine serum albumin (Rogalinski et al., 2005). These authors also determined arginine as one of the most abundant free amino acids in the subW hydrolysates, unfortunately, arginine can not be determined wit the EZ:Faast kit used in this work. The high content of these simple amino acids has been attributed to their stability under the working conditions, but also to the possibility of formation from decomposition of complex amino acids to simpler ones (Koh et al., 2019; Rogalinski et al., 2005). Production of these low molecular weight amino acids is important since they can be used as sweetness enhancing agents for use as food additives and taste enhacers (Quitain et al., 2001).

Fig. 3 shows the recovery yield of each individual amino acid determined as:

$$Y_i = m_{i,subW}/m_{i,WSP} \quad [3]$$

where $m_{i,subW}$ is the amount of amino acid i determined in the subW hydrolysates and $m_{i,WSP}$ the total amount of the amino acid i in the initial raw material, WSP, including the free and bound amino acids. Global yield, evaluated by considering the sum of all the individual free amino acids, has been also evaluated and listed in Table 1. Recovery yield of total free amino acids increased with temperature and in the presence of CO_2, observing significant differences at the different temperatures essayed and between both pressurization agents, reaching a maximum value of 34 ± 3 % at 180 °C.

According to the results presented in Fig. 3, the highest recovery yield was achieved by alanine followed by glycine, achieving more than 80 % of alanine recovery at 180 °C in the presence of CO_2 (more than 70 % in N_2). Appart from alanine and glycine, as a general trend, the highest values of the individual recovery yield were obtained for most of the hydrophobic amino acids (leucine, phenylalanine, proline and valine) and the sulphur containing amino acid metionine. For these amino acids, there was a clear trend of increasing recovery yield by increasing temperature. This trend was also found by Hao et al. (2019) in the subW hydrolysates of Abalone viscera; however, for other free amino acids there was no trend with temperature or even a slightly decrease by increasing the working temperature (isoleucine, serine, threonine, serine or aspartic acid). Therefore, as concluded by Esteban et al. (2010), although some trends could be determined according to the type of side chain of the amino acid, not all the amino acids considering a specific side chain type were obtained in proportional amounts according to the amino acid composition of the protein source.

The kinetics of the release of total and individual free amino acids from the WSP extract in subW have been determined at 180 °C in the presence of CO_2 and N_2. Some of the most representative free amino acids have been represented in Fig. 4a and 4b for N_2 and CO_2, respectively. In these figures, the amount of free amino acids at zero treatment time corresponds to the free amino acids that are initially soluble in water.

The qualitative profile for free amino acids was similar for CO_2 and N_2 pressurization agents; however, for total free amino acids and most of the major free amino acids it was observed a linear increase with reaction time in the presence of CO_2 suggesting that the maximum yield

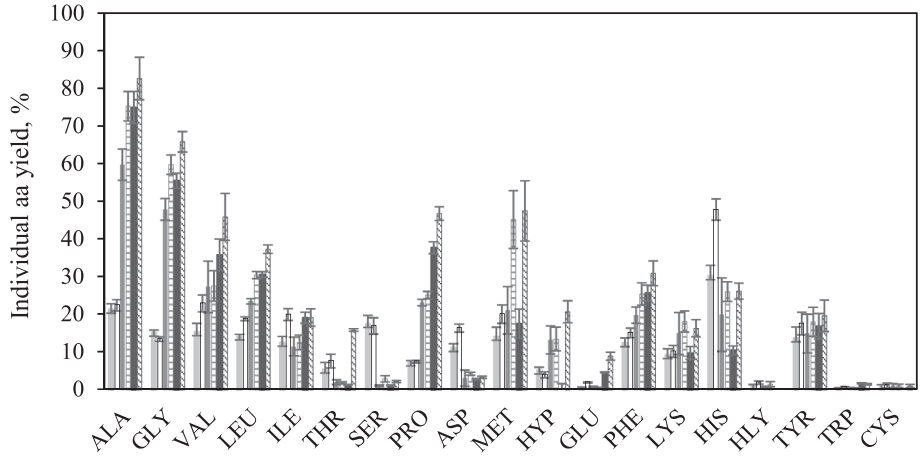

Fig. 3. Individual amino acid yield obtained by subW with different pressurization agents and temperatures. (140 °C: ▫ N_2, □ CO_2) (160 °C: ■ N_2, ▤ CO_2) (180 °C: ■ N_2 ▧ CO_2) after 300 min treatment.

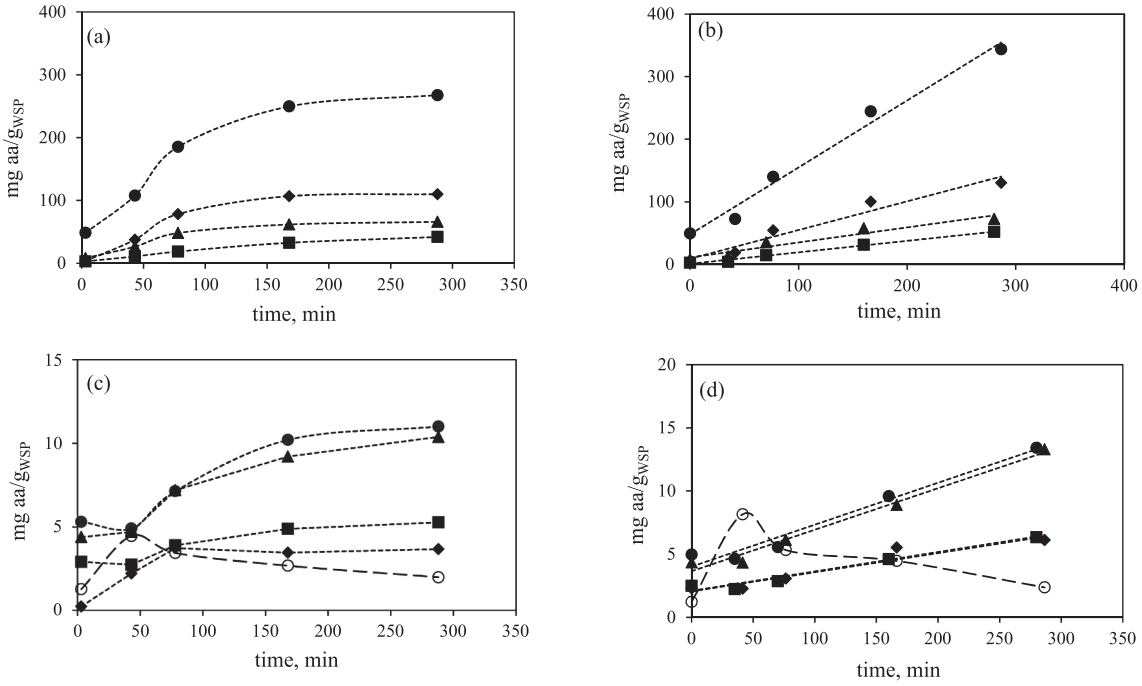

Fig. 4. Total and individual amino acids release kinetic by subW at 180 °C. Total and major free amino acids (a) in N_2 (b) in CO_2: ● total amino acids, ◆ glycine, ▲ alanine, ■ proline. Other amino acids: (c) in N_2 (d) in CO_2: ● leucine, ▲ valine, ■ phenylalanine, ◆: lysine, ○ aspartic acid.

could not have been achieved. On the contrary, in the presence of N_2, there was a continuous increase with hydrolysis time up to 170 min and afterwards a plateau was reached and no further hydrolysis was taking place. These results also indicated that the predominant free amino acids were stable under the employed conditions.

Fig. 4c and 4d also show the evolution of aspartic acid. Although aspartic acid was not one of the major free amino acids determined in subW hydrolysate of WSP, it has been represented to show its maximum peak in the first minutes of treatment time and then a continuous decrease with reaction time. In this regard, it must be highlighted that aspartic acid belongs to the amino acid class of negatively charged group that has been found to be very liable in the hydrolysis of other protein sources such as BSA (Abdelmoez and Yoshida, 2013) or vegetable proteins (Alonso-Riaño et al., 2021).

3.3.4. *Antioxidant capacity of subW hydrolysates*

The antioxidant capacity of the subW hydrolysates of WSP was determined along treatment time and results are presented in Fig. 2e and 2f in the presence of N_2 and CO_2, respectively. Reducing capacity at zero treatment time corresponds to the reducing power of the WSP extract with a value of 0.569 ± 0.002 mM Fe^{2+}.

All WSP hydrolysates showed an increasing reducing capacity due to subW treatment and it increased with operation temperature. Correlation coefficients between the reducing capacity of the hydrolysates and the amino group released have been determined according to the Pearson product moment correlation. Correlation has been also

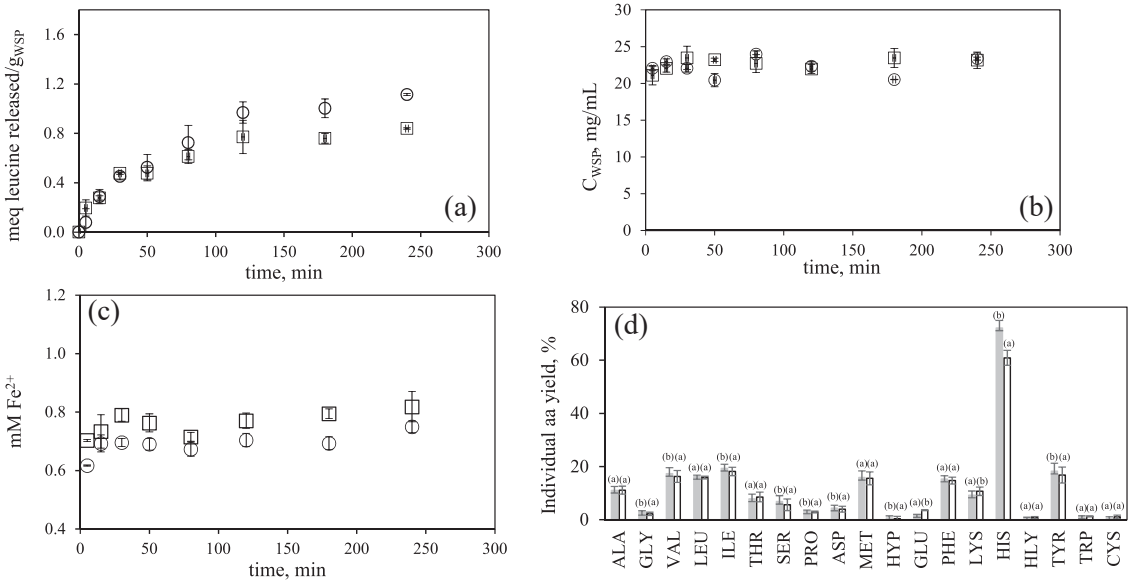

Fig. 5. Enzymatic hydrolysis of WSP at 60 °C and pH = 8 and 225 U/100 mL of WSP extract (a) amino group released (b) total protein content (c) reducing capacity: ○ Alcalase® □ Novozym® (d) Individual amino acid yield: ■ Alcalase®, □ Novozym®.

established between the reducing capacity and the results obtained by the Lowry assay since the decrease in the total protein content (Fig. 2c and 2d) might indicate formation of smaller peptides with higher antioxidant capacity. Analysis indicated a statistically significant non-zero correlations at the 95.0% confidence level coefficients between both variables with positive and negative correlation coefficients between reducing capacity and amino group release and Lowry assay, respectively. Correlation coefficients were 0.9316, −0.8624 for amino group release and Lowry assay, respectively in the presence of CO_2 (n = 35, the number of pairs of data values used to compute each coefficient) and 0.9159, −0.9238 for NH_2 release and Lowry assay, respectively in the presence of N_2 (n = 30). Based on these coefficients, the strength of the correlation between reducing capacity and amino groups and the Lowry assay, as an indicator of smaller peptides size production, was of the same order.

3.4. Enzymatic hydrolysis of WSP

The results obtained in the hydrolysis of WSP by subW have been compared with conventional enzymatic hydrolysis by using two different commercial proteases, Alcalase® and Novozym®. Fig. 5 a, b and c show the amino group release, the total protein content and the reducing activity obtained along time, respectively.

For both enzymes, the release of $–NH_2$ group is much lower than the release observed in subW, less than 1.2 meq leucine/g WSP were released after 240 min by enzymatic hydrolysis. By comparing both enzymes, final significant higher $–NH_2$ group release was observed for Alcalase® than for Novozym® (nearly a 30 % higher), although there was not observed a significantly higher initial $–NH_2$ group release for both enzymes (Fig. 5a).

Regarding total protein content, a clear decrease was observed by subW hydrolysis attributed to the production of small peptides and free amino acids; however, not a clear trend was observed in the total protein content by enzymatic hydrolysis that could be attributed to the generation of peptides of bigger size than by subW hydrolysis as it will be presented in section 3.5 (see Fig. 5b). Furthermore, there was no statistically significant differences between the medium total protein content both both enzymes along the kinetic process.

Fig. 5c shows an initial slight increase in the reducing capacity of the enzymatic hydrolysates for both enzymes. The medium value of reducing capacity along enzymatic treatment was significantly higher for Novozyme® than for Alcalase®; however, the reducing activity, for both enzymes, was much lower than the one observed by subW hydrolysis.

Total and individual free amino acids were also determined in the final enzymatic hydrolysates with similar values for both enzymes (75 ± 1 and 71.0 ± 0.6 mg/g WSP for Alcalase and Novozym, respectively) but much lower than the amount of free amino acids generated by subW hydrolysis. Table 1 shows the individual amino acid profile expressed as mg aa/g WSP obtained by Alcalase® and Novozym®, observing a similar free amino acid profile for both enzymes. The highest concentration corresponds to histidine (19.0 ± 0.7 and 16.1 ± 0.2 mg aa/g WSP for Alcalase® and Novozym®, respectively) followed by alanine (9.7 ± 0.1 and 9.8 ± 0.1 mg aa/g WSP for Alcalase® and Novozym®, respectively). The yield of the individual free amino acids was also evaluated according to Equation 4 and results are presented in Fig. 5d. The highest hydrolysis yield was obtained for histidine, with values of 75 ± 1 % and 71 ± 0.1 %, for Alcalase® and Novozym®, respectively. For the rest of amino acids, hydrolysis yields were lower than 20 %. It must be highlighted the lower yield obtained for glycine, less than 3 %, being this small size amino acid one the major free amino acid obtained in subW hydrolysates. Both commercial enzymes are sold as endopeptidases, which may explain the low production of free amino acids. Comparison with literature data regarding the free amino acid profile after enzymatic hydrolysis of marine protein is difficult since most of the studies do not present the individual free amino acid profile. Ovissipour et al. (2009) reported individual free amino acid yields after enzymatic hydrolysis of Persian sturgeon viscera by Alcalase® yielding the highest value for arginine (93 %), histidine (79 %) and methionine (75 %). The yield of histidine was of the same order as the one obtained in this work; unfortunately, arginine could not be determined by the analytical methods used in this work.

3.5. Molecular weight distribution of WSP hydrolysates

Molecular weight of the protein hydrolysates will play an important role in their functional properties. Therefore, to obtain information about the hydrolysate-length distribution and variations according to the different hydrolysis treatments, final subW hydrolysate obtained by subW in the presence of CO_2 at 180 °C and the enzymatic hydrolysate obtained by Alcalase® were analysed by size-exclusion chromatography, and the results are shown in Fig. S2, together with the molecular weight distribution of the WSP extract.

Three major fractions were distinguished in the chromatogram presented in Fig. S2: fraction I corresponds to the higher molecular size distribution proteins and peptides (greater than17 kDa), fraction II corresponds to the peptides with molecular sizes around 0.4–0.5 kDa, whereas fraction III corresponds to the lower molecular ranged determined by the equipment configuration, less than 0.1 kDa.

Both hydrolysis treatments, subW and enzymatic reduced the peptide size lengths compared with those of the original WSP extract. It was observed that the presence of the highest molecular weight species (fraction I) in the samples was more abundant in WSP, followed by Alcalase® hydrolysate and subW hydrolysate. These results support previous results presented in this work, in which the high molecular weight fraction present in the WSP extract was reduced after the enzymatic treatment and even more after the subW treatment. The hydrolysis was more intense in the subW hydrolysate, which showed more minor peaks in fraction III than WSP extract and the enzymatically hydrolyzed sample. The molecular weight distribution obtained in Fraction III agreed with the results presented in Table 1 where the total amount of free amino acids released by subW in CO_2 at 180 °C was higher than the amount of free amino acids released by enzymatic treatment or the free amino acid present in the WSP extract. Molecular weight of amino acids ranges from 75 Da for glycine ($C_2H_5NO_2$) to 204 Da for tryptophan ($C_{11}H_{12}N_2O_2$). Therefore, the higher distribution obtained for subW hydrolysate in Fraction III would correspond to the higher amount of free amino acids generated by subW (344 ± 5 mg aa /g WSP at 180 °C in CO_2) compared to enzymatic hydrolysates (71.0 ± 0.6 mg free aa/g WSP for Alcalase®). Similar distribution of the lowest molecular weight range (Fraction III) was obtained for the WSP extract and the enzymatic hydrolysates supporting by the similar amount of free amino acids in both samples (see Table 1).

It is also worth highlighting that the signals of peptides of Fraction II was much higher for subW hydrolysate than for the enzymatic hydrolysate. Taking into account the molecular weight distribution of individual amino acids (from 75 Da to 204 Da), the small peptides generated in this fraction would consist of 2–6 amino acids units. Fraction II was more prominent in the subW hydrolysate supporting the fact that smaller peptides were generated than by enzymatic hydrolysis. These results were supported by the Lowry assay that might indicate the generation of smaller size peptides by subW than by enzymatic hydrolysis due to the decreasing signals as decreasing the peptide molecular size.

4. Conclusions

Protein hydrolysates from fish meal can be an excellent source of nitrogen for different food applications. The combination of analytical techniques performed in this work, such as the amino group release, protein content by Lowry method, (free and bound) amino acid profile, antioxidant capacity and molecular weight distribution, has been

carried out in order to characterize the protein hydrolysis and explore alternatives to the conventional process.

According to the amino group release profiles and the production of free amino acids, temperature in the 140–180 °C range has a positive effect in the WSP hydrolysis by subW. The use of CO_2 as pressurization agent favoured the production of free amino acids, likely due to the carbonate formation and the associated pH reduction of subW, compared to the inert N_2. The Lowry analysis showed lower responses when increasing hydrolysis temperature and treatment time, which is likely related to the reduction in peptide size and the liberation of non-reactive free amino acids, rather than to protein degradation. The molecular weight distribution supported the results obtained in the amino group release and free amino acid analyses.

Compared to enzymatic hydrolysis with commercial proteases, hydrolysis of the WSP fraction of fish meal by subW achieved much higher release of amino groups and free amino acids in a similar reaction time, and with no need of additional catalysts or reagents. Small amino acids such as glycine and alanine were preferentially released by subW treatment compared to enzymatic hydrolysis. These results show the benefits of subW technology for protein valorization.

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CRediT authorship contribution statement

P. Barea: Methodology, Data curation, Investigation. **R. Melgosa:** Conceptualization, Writing – review & editing. **A.E. Illera:** Data curation, Methodology. **P. Alonso-Riaño:** Methodology. **E. Díaz de Cerio:** Methodology. **O. Benito-Román:** Methodology, Data curation. **Sagrario Beltrán:** Writing – review & editing, Funding acquisition. **M. Teresa Sanz:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.135925.

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