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Maximizing hypocholesterolemic peptides from an olive byproduct by enzymatic hydrolysis

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ABSTRACT

Novel food proteases (Maxipro A, Maxipro B, Maxipro C, and Maxipro D) with broad specificity and different effective pH range (from 2.5 to 9.0) were evaluated to produce hypocholesterolemic peptides from olive seeds proteins. Box-Behnken experimental design was employed to evaluate the effect of the hydrolysis time, pH, temperature, and enzyme:substrate ratio on the amount of released peptides. The production of peptides, under optimal conditions, was higher with Maxipro C and lower with Maxipro B. The capability of extracts to reduce the absorption of exogenous cholesterol and the production of endogenous cholesterol was also evaluated. All hydrolysates showed a higher capacity to bind bile acids and inhibit cholesterol esterase enzyme than the obtained with usual Alcalase enzyme. Proteomic analysis using high resolution RP-HPLC-ESI-QTOF with database searching and *de novo* identification was employed to identify peptides in hydrolysates. Some sequences with hypocholesterolemic features were detected among the 45 peptides identified in Maxipro A hydrolysate, the 56 peptides in that of Maxipro C, and the 50 in the Maxipro B one. This work demonstrates that Maxipro A enzyme can hydrolyze olive seeds proteins, under acidic pH, releasing peptides with multifunctional and higher hypocholesterolemic capacity than the obtained with Alcalase, under alkaline conditions.

1. Introduction

Bioactive peptides are organic substances, which show benefits on health and exert biological activities such as antioxidant, antihypertensive, or hypocholesterolemic, among others (Sánchez & Vázquez, 2017). These molecules use to be fragments of proteins with 2–20 amino acids length. The most common methods to obtain bioactive peptides from parent proteins are enzymatic hydrolysis, microbial fermentation, or chemical hydrolysis (Jakubczyk, Karaś, Rybczyńska-Tkaczyk, Zielińska, & Zieliński, 2020; Ulug, Jahandideh, & Wu, 2021). Enzymatic hydrolysis is the preferred way to produce bioactive peptides due to its high specificity and because it does not need to use organic solvents (Zambrowicz, Timmer, Polanowski, Lubec, & Trziszka, 2013). However, this process requires to work under controlled conditions (pH, temperature, time of hydrolysis, and enzyme concentration) and that, a previous evaluation of promoting hydrolysis conditions is always needed.

Hypercholesterolemia is a metabolic disorder and it is associated with a high cardiovascular disease risk. This disorder is characterized by a high blood cholesterol level. There are two sources of cholesterol: endogenous cholesterol, which is synthesized by the organism, and exogenous cholesterol, which is obtained through the diet. Different synthetic drugs are commercially available to reduce blood cholesterol level by inhibiting cholesterol synthesis or exogenous cholesterol absorption. Nevertheless, the use of these synthetic molecules can result in undesirable side effects (digestive disturbances, nausea, increase of hepatic transaminases and creatine kinase, muscle weakness, headache, etc.) and, thus, the search for alternative natural compounds to treat or prevent hypercholesterolemia is highly interesting (Singh & Sashidhara, 2017). Among natural compounds, some bioactive peptides have been reported to have a great capacity to reduce both endogenous and exogenous cholesterol (Prados, Orellana, Marina, & García, 2020; Wergedahl et al., 2004).

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Different proteases have been employed to obtain bioactive peptides with hypocholesterolemic capacity. Alcalase has demonstrated to be capable to release hypocholesterolemic peptides from different proteins such as olive seed proteins (Prados, Plaza, Marina, & García, 2020; Prados, Marina, & García, 2018), chia proteins (Coelho, Soares-Freitas, Arêas, Gandra, & Salas-Mellado, 2018), camel mik proteins (Mudgil et al., 2019) or soy proteins (Lammi, Zanoni, & Arnoldi, 2015). Other enzymes less employed for this purpose are thermolysin or flavourzyme. Moreover, the use of digestive enzymes is also usual since they release peptides during human digestion. As example, trypsin has been used to obtain hypocholesterolemic peptides from bovine milk (Nagaoka et al., 2001).

Our research group has demonstrated by *in vitro* and *in vivo* methods that olive seeds are a natural source of peptides with capacity to reduce cholesterol by the increase of blood high-density lipoproteins (Prados, Orellana, et al., 2020; Prados et al., 2018). Two different enzymes were evaluated (Alcalase and Thermolysin) but commercial Alcalase enzyme was the best protease for this purpose (Prados, Plaza, et al., 2020). Further studies using chemometric tools have also stated that peptide NFVVLK, in olive seeds hydrolysates, displayed a strong correlation with the capacity to reduce exogenous cholesterol absorption, while the presence of peptide WVAF was associated with the capacity to reduce endogenous cholesterol (Prados, Plaza, et al., 2020). On the other hand, the growing demand for novel enzymes by the food industry (e.g., preparation of hypoallergenic food, wine, beer, and juice production, meat tenderization, baking and cheese preparations, removal of flavour and pigments, etc.) has promoted the development of new enzymes with enhanced abilities. These enzymes might also have capability to release hypocholesterolemic peptides and could be especially useful in cases where is not possible to work with Alcalase enzyme.

The aim of this work was to explore the potential of four novel proteases (Maxipro A, Maxipro C, Maxipro B, and Maxipro D) to release peptides with hypocholesterolemic features from olive seeds and to identify potential bioactive peptides. Results will be compared with the obtained by using Alcalase enzyme, usually employed for producing bioactive peptides.

2. Materials and methods

2.1. Materials

All chemicals and reagents were of analytical grade. Water was daily obtained from a Milli-Q system from Millipore (Bedford, MA, USA). Acetic acid (AA), acetone, acetonitrile (ACN), hexane, and methanol were purchased from Scharlau Chemie (Barcelona, Spain). Di-sodium tetraborate, hydrochloric acid, sodium dihydrogen phosphate, sodium dodecyl sulphate (SDS), and Tris (hydroxymethyl) aminomethane (Tris) were from Merck (Darmstadt, Germany). Bovine pancreatic cholesterol esterase, cholesterol, dithiothreitol (DTT), HMG-CoA R (3-hydroxy-3-methylglutaryl-coenzyme A reductase) assay kit, β-mercaptoethanol, *p*-nitrophenylbutyrate (*p*-NPB), oleic acid, phosphatidylcholine, *o*-phthaldialdehyde (OPA), sodium chloride, and taurocholic acid, taurodeoxycholic acid were all from Sigma-Aldrich (Saint Louis, MO, USA). Cholesterol oxidase kit was purchased from BioAssay Systems (Hayward, CA, USA). Total bile acid kit was from Bio-Quant (San Diego, CA, USA). HMG-CoA R assay kit was purchased in Sigma-Aldrich.

The four proteases employed in this work (Maxipro A, Maxipro C, Maxipro B, Maxipro D) were kindly donated by DSM (Heerlen, The Netherlands). Raw olives of Picual variety were kindly donated by the World Olive Germplasm Bank of IFAPA (Córdoba, Spain). Olives were collected at violet maturity index.

2.2. Extraction of proteins from olive seed

Olives were manually depulped and stones were stored at −20 °C until use. Seeds inside stones were extracted manually and ground in a domestic miller. Seed powder was defatted and proteins were extracted using the method described by Esteve, Del Rio, Marina, and García (2010). Briefly, 0.03 g of samples were mixed with 5 mL of extracted buffer (0.1 mol/L Tris-HCl, 0.5 g/100 mL SDS and 0.5 g/100 mL DTT, pH 7.5) using a high intensity focused ultrasound (HIFU) probe (model VCX130, Sonic Vibra-Cell, Hartford, CT, USA) at 30% of amplitude for 5 min. After centrifugation (6000×*g*), proteins in the supernatant were collected and precipitated with cold acetone (ratio 2:1). Isolated proteins were stored at – 20 °C until use.

2.3. Production of hydrolysates

Protein isolate was dissolved in 5 mmol/L borate or phosphate buffer at different pHs previously to hydrolysis with Maxipro A, Maxipro C, Maxipro B, and Maxipro D (DSM, Netherlands). Hydrolysis with Alcalase was carried out according to Prados et al. (2018). Briefly, protein isolate was dissolved in a 5 mmol/L borate buffer (pH 8.5) using HIFU. After addition of enzyme (0.15 UA/g proteins), the mix was incubated at 50 °C during 4 h. In all cases, digestion was stopped by increasing the temperature until 100 °C for 10 min. Finally, the hydrolysate was centrifuged for 10 min at 6000×*g* and the supernatant was collected.

Hydrolysis conditions with Maxipro enzymes (temperature, pH, time, and enzyme:substrate ratio) were optimized using experimental design. The Box–Behnken design was carried out using Statgraphics Software Plus 5.1 (Statpoint Technologies, Inc., Warranton, VA, USA). The response variable was the content of peptides estimated by the OPA method (Wang et al., 2008), after subtracting the contribution of the own enzymes. Experimental data were fitted to a quadratic model using a second-order polynomial model equation:

$$Y\ (\text{response variable}) = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

where β_0 is the constant, β_i is the linear regression coefficient, β_{ii} is the quadratic regression coefficient, and β_{ij} is the interaction regression coefficient, while X_i and X_j are the independent variables. The fitting of data to this polynomial model equation was evaluated using the determination coefficient (R^2) and the analysis of variance (ANOVA), at a confidence level of 95%.

2.4. Evaluation of the hypocholesterolemic capacity

Hypocholesterolemic activity was evaluated by four different assays. Three of them were based on mechanisms to reduce exogenous cholesterol absorption (reduction of micellar cholesterol solubility, cholesterol esterase enzyme inhibition, and binding of bile acids) and the fourth one evaluated the capacity to reduce the synthesis of endogenous cholesterol (HMG-CoA R inhibition). These assays have been described in previous works of our research group (Prados, Orellana, et al., 2020; Prados et al., 2018). Briefly, micellar cholesterol solubility assay evaluated the capacity of hydrolysates to disrupt micelles solubilizing cholesterol. Artificial micelles containing cholesterol were prepared by mixing 0.5 mmol/L cholesterol, 1 mmol/L oleic acid, 2.4 mmol/L phosphatidylcholine, 15 mmol/L PB (pH 7.4), 6.6 mmol/L taurocholate salt, and 132 mmol/L NaCl. After incubation of hydrolysates with micelles for 2 h at 37 °C, the amount of cholesterol remaining in micelles was determined spectrophotometrically using a cholesterol commercial kit. The cholesterol esterase enzyme inhibition assay evaluated the capacity of hydrolysates to inhibit that enzyme that hydrolyses dietary cholesterol esters into free cholesterol. This capacity was determined by the inhibition of the cholesterol esterase enzyme when catalysing the hydrolysis of *p*-NPB to *p*-nitrophenol and measuring its absorbance. The capacity of hydrolysates to bind to bile acids and promoting their synthesis in the liver by oxidation of endogenous cholesterol was evaluated by the incubation of hydrolysates with 2 mmol/L bile acids (37 °C for 90 min) and determining solubilized bile acids using a commercial kit. Finally, the HMG-CoA R inhibition assay evaluated the capacity to avoid

endogenous cholesterol synthesis by the catalysis of the reaction of HMG-CoA to mevalonate. This assay was carried out using a commercial kit.

2.5. Separation and identification of peptides by RP-HPLC-MS/MS

The separation and identification of peptides present in the hydrolysates obtained with different enzymes was carried out using a High-Performance Liquid Chromatography (HPLC) system 1100 from Agilent (Agilent Technologies, Santa Clara, CA, USA) coupled to a high sensitive Quadrupole-Time-of-Flight mass spectrometer (Q-TOF/MS) (Agilent 6530 series, Pittsburgh, PA, USA) equipped with an orthogonal electrospray ionization (ESI) source (Agilent Jet Stream, AJS). Agilent Mass Hunter Workstation software B.07.00 was used for HPLC and MS control, data acquisition, and data analysis. Separation was performed in an Ascentis Express Peptide ES-C18 column (100 × 2.1 mm, 2.7 μm particle size) with an Ascentis Express Peptide ES-C18 guard column (5 × 2.1 mm, 2.7 μm particle size), both from Supelco (Bellefonte, PA, USA). The methodology was very similar to the used in a previous work (Prados, Orellana, et al., 2020), except for the elution gradient. The mobile phases were water with 0.3% acetic acid (v/v) (phase A) and acetonitrile with 0.3% acetic acid (v/v) (phase B). The elution gradient was: 5% B for 5 min, 5–45% in 35 min, 45–90% B in 2 min, 95% for 3 min. This separation gradient was followed by a step to return to initial conditions from 95 to 5% B in 5 min and 20 min of post-time. The injection volume was 15 μL and the flow rate was 0.3 mL/min. MS detection was performed in the positive ion mode using a mass range from 100 to 1500 m/z. MS conditions were: fragmentator voltage 200 V; nebulizer pressure, 50 psig; capillary voltage, 3500 V; gas temperature, 350 °C; drying gas flow, 12 L/min; skimmer voltage, 60 V; and octapole voltage, 750 V. The Jet Stream sheath gas conditions were: 12 L/min and 400 °C. MS/MS was carried out using *Auto* mode with the following conditions: 5 precursors per cycle and a collision energy of 5 V per each 100 Da. Two Agilent compounds (HP0921 and purine) yielding ions at 922.0098 m/z and 121.0509 m/z, respectively, were simultaneously introduced as internal standards throughout the analysis.

Data obtained by RP-HPLC-MS/MS were exported to PEAKS Studio software from Bioinformatics Solutions Inc. (Waterloo, Canada) for the *de novo* sequencing of peptides. In addition, data were analysed by PEAKS DB (database search tool) using FASTA database that included protein sequences from *Olea Europaea* organism obtained from UniProt database.

For peptides identified using FASTA database, peptides sequences were associated to a protein if the error tolerance was <10 ppm and the mass tolerance was 0.5 Da for the fragments. Peptides with a −10lgP equal or higher to 15 confirmed the confidence between them.

For peptides identified using *de novo* sequencing, only peptides appearing in all injections (six injections, three injections of each extract) with an ALC (expected percentage of correct amino acids in the peptide sequence) above 90% were considered. Since it is not possible to make differences between isoleucine (I) and leucine (L) amino acids, only isoforms with L were displayed, although peptide sequences containing I amino acid instead of L are also possible. Moreover, isoelectric points and water solubility of identified peptides were calculated using Innovagen peptide property calculator (https://pepcalc.com). Functionality of proteins was obtained from QuickGo (www.ebi.ac.uk/QuickGo).

2.6. Statistical analysis

Statistical analysis was performed using Statgraphics Software Plus 5.1 (Statpoint Technologies, Inc., Warranton, VA, USA). Data comparison was carried out by the analysis of variance (ANOVA). Duncan's Multiple Range test was used to determine statistically significant differences ($P < 0.05$). Results obtained were expressed as mean ± standard deviation.

3. Results and discussion

Previous works of our research group employed Alcalase enzyme to obtain peptides with hypocholesterolemic capacity from olive seeds (García, González-García, Vásquez-Villanueva, & Marina, 2016; Prados, Orellana, et al., 2020; Prados, Plaza, et al., 2020; Prados et al., 2018). Alcalase is an alkaline enzyme (pH 7–10) from *Bacillus licheniformis* with a high efficiency in hydrolysing proteins. This work will compare the performance of this enzyme to release peptides with hypocholesterolemic characteristics with four novel proteolytic enzymes with broad specificity whose characteristics are included in Table 1. Maxipro A is derived from *Aspergillus niger* and, unlike Alcalase, it shows activity at very acid pHs. Maxipro B, from *Aspergillus oryzae*, and Maxipro D, derived from *Bacilus amyloliquefaciens*, show high activity at slightly acid pHs, while Maxipro C, derived from *Bacillus licheniformis*, is, as Alcalase, an alkaline protease. Since this is the first time that these enzymes were employed to hydrolyze olive seed proteins, it was necessary a previous optimization of hydrolysis conditions.

3.1. Optimization of hydrolysis conditions

A Box-Behnken experimental design was employed to optimize the following hydrolysis parameters with every enzyme: temperature, pH, time, and enzyme:substrate ratio. The enzyme:substrate ratio and the time ranged from 0.1 to 1.0 UA/g protein and from 1 to 24 h, respectively, for all enzymes. Nevertheless, the range of temperature and pH depended on every enzyme and was in agreement with their characteristics (see Table 1). Experimental design established thirty experiments, for every enzyme, that were conducted in a randomized order: twenty-four points corresponded to the factorial design and six centre points were considered to evaluate experimental errors. Experimental conditions in these experiments and peptide content (corrected by subtracting the contribution of every enzyme) in resulting hydrolysates are grouped in Tables S1–S3, respectively. Hydrolysis of olive seed proteins using Maxipro D enzyme required a very high amount of enzyme and therefore, it was discarded.

Hydrolysis with Maxipro A was promoted at low pHs and long hydrolysis times, while the effect of temperature was very subtle and the effect of the enzyme:substrate ratio was neglected. The highest peptide content (1.89 mg/mL) was observed in experiment 22 (45 °C, pH = 2.5, 24 h, and enzyme:substrate ratio of 2.75 UA/mg protein) and the lowest (0.59 mg/mL) in experiment 7 (45 °C, pH = 4.0, 1 h, and enzyme:substrate ratio of 5 UA/mg protein). The reproducibility of central points (experiments 3, 6, 8, 12, 15, and 25) was 20% with this enzyme. Hydrolysis with Maxipro C was highly enhanced at long times and enzyme:substrate ratios while the effect of temperature and pH was less significant. The highest peptide content (2.71 mg/mL) was observed in experiment 18 (50 °C, pH = 9.0, 24 h, and enzyme:substrate ratio of 2.75 UA/mg protein) and the lowest (0.78 mg) in experiment 1 (35 °C, pH = 8.25, 12.5 h, and enzyme:substrate ratio of 5 UA/mg protein). The reproducibility of central points (experiments 3, 11, 12, 19, 25, and 27) was 12% with this enzyme. Hydrolysis with Maxipro B was improved at high pHs while the effect of temperature, time and substrate:enzyme ratio were lower. The highest peptide content (1.11 mg/mL) was observed in experiment 11 (50 °C, pH = 7, 12.5 h, and an enzyme:substrate ratio of 2.75 UA/mg protein) and the lowest (0.56 mg/mL) in experiment 25 (35 °C, pH = 6.0, 24 h, and enzyme:substrate ratio of 2.75 UA/mg protein). The reproducibility of central points (experiments 6, 14, 15, 18, 19, and 28) was 11% with this enzyme. Table 2 shows the coefficients of the multiple linear regression model best fitting the peptides content for the three enzymes. Moreover, it also shows the analysis of variance and the determination coefficient. The three mathematical models were capable to explain 71–82% of the peptide content variability. Moreover, ANOVA demonstrated that established models were suitable (P (lack of fit) > 0.05). According to the mathematical models, variables most affecting the release of peptides (P <

Table 1
Working temperature and pH ranges and source organism and activity of enzymes employed to hydrolyze olive seed proteins.

Enzyme	Maxipro A	Maxipro B	Maxipro C	Maxipro D	Alcalase
Temperature range (°C)	35–55	35–50	35–65	35–50	30–65
pH range	2.5–5.0	5.5–7.0	7.5–9.0	5.5–7.0	7.0–10.0
Source organism	*Aspergillus niger*	*Aspergillus oryzae*	*Bacillus licheniformis*	*Bacillus amyloliquefaciens*	*Bacillus licheniformis*
Activity	1.000–1.100 SAPU/g	Not provided	≥480 DAPU/g	≥180000.0 PC/g	2.4 AU/g

SAPU: Spectrophotometric Acid Protease Unit.
DAPU: Detergent Alkaline Protease Unit.
PC: Protease Unit on L-Tyrosinase Basis.
AU: Aston unit.

Table 2
Coefficients and ANOVA of the multiple linear regression models obtained in the hydrolysis of olive seed proteins using Maxipro enzymes.

Parameters	Maxipro A		Maxipro C		Maxipro B	
	Coefficients	*p*-value	Coefficients	*p*-value	Coefficients	*p*-value
Constant	1.7897		12.0215		5.0925	
A	0.0155	0.8122	−0.0300	0.0734	−0.0913	0.0621
B	−0.7898	0.0105	−2.4998	0.2911	−0.9298	0.0328
C	0.0732	0.0058	0.1028	0.0032	0.0079	0.2645
D	−0.0117	0.9609	−0.4344	0.0327	0.0008	0.6682
AA	−0.0004	0.5652	−0.0016	0.0158	−0.0001	0.8886
AB	0.0037	0.5386	0.0236	0.0924	0.0164	0.0351
AC	0.0001	0.8832	−0.0013	0.1422	0.0003	0.5610
AD	0.0010	0.8041	0.0065	0.1452	0.0015	0.5743
BB	0.0869	0.0286	0.0755	0.6820	0.0272	0.4449
BC	−0.0140	0.0351	0.0004	0.9779	−0.0001	0.9876
BD	−0.0095	0.7193	0.0671	0.4167	−0.0076	0.7083
CC	−0.0002	0.7033	−0.0001	0.8681	−0.0009	0.0148
CD	0.0007	0.8346	−0.0016	0.7522	−0.0003	0.8692
DD	−0.0004	0.9759	−0.0599	0.0268	−0.0021	0.7547
R^2	0.8208		0.7141		0.7935	
p-value (lack of fit)	0.6776		0.1574		0.8020	

A: Temperature; B: pH; C: Time; D: Enzyme:substrate ratio.

0.05) were the pH and incubation time, in the case of Maxipro A, the time and the enzyme:substrate ratio for Maxipro C, and the pH for Maxipro B. Fig. 1 shows the effect of variables on the peptide content of hydrolysates as 3D contour plots, at different enzyme:substrate ratios. Contour plots clearly showed that the effect of the enzyme:substrate ratio for Maxipro A and Maxipro B enzymes was negligible but very important in the case of the Maxipro C. Furthermore, the most influencing variable in the hydrolysis with Maxipro A and Maxipro C enzymes was the incubation time while for Maxipro B was pH. These results are in agreement with the observed from data included in Tables S1–S3. Finally, Table 3 groups optimal conditions for the hydrolysis of olive seed proteins with the three enzymes, the estimated peptides content, and the peptide content determined experimentally in the hydrolysates obtained under optimal conditions. Peptide content obtained with every enzyme was significantly different observing the highest hydrolysis degree in the hydrolysate corresponding to Maxipro C followed by those from Maxipro A and Maxipro B. No significant difference was observed between the peptide contents predicted by the mathematical model and the observed in the hydrolysates, which confirmed the suitability of these mathematical models.

3.2. Evaluation of the hypocholesterolemic activity of hydrolysates

The hypocholesterolemic capacity of hydrolysates obtained using the optimal conditions for the three enzymes was evaluated by four different *in vitro* hypocholesterolemic assays related with the capacity to reduce diet and endogenous cholesterol: capacity to bind bile acids, capacity to inhibit the activity of cholesterol esterase enzyme, ability to reduce the micellar cholesterol solubility, and capacity to inhibit HMG-CoA R enzyme. Fig. 2 shows the obtained results in comparison with the observed when using Alcalase enzyme (Prados, Plaza, et al., 2020). Bile acids are synthesized in the liver from endogenous cholesterol, thus, sequestering of bile acids results in a decreasing cholesterol level. Bile acids binding capacity of hydrolysates obtained from all Maxipro enzymes was much higher than the observed in the hydrolysate obtained with Alcalase. On the other hand, cholesterol esterase enzyme hydrolyses dietary cholesterol esters into free cholesterol, promoting its bioavailability. All Maxipro enzymes led to hydrolysates with a high capacity to inhibit cholesterol esterase while the Alcalase hydrolysate did not show any capacity. The highest inhibition of cholesterol esterase was observed in the hydrolysate obtained with Maxipro A. Moreover, diet cholesterol requires to be solubilized in micelles for its transportation through a polar medium and, thus, the inhibition of this process is a mechanism to avoid cholesterol absorption. All hydrolysates obtained with Maxipro enzymes showed capacity to reduce micellar cholesterol solubilisation, although this capacity was not as high as the yielded by the Alcalase hydrolysate. Finally, the capacity of hydrolysates to avoid the production of endogenous cholesterol was evaluated by studying their capacity to inhibit HMG-CoA R enzyme. This enzyme catalyses one of the steps happening in the biosynthesis of cholesterol, the transformation of HMG-CoA to mevalonate. Again, the hydrolysate obtained with Maxipro A yielded the highest inhibition. According to these results, Maxipro A is a promising enzyme to obtain multitask hypocholesterolemic peptides and an alternative to Alcalase. Unlike Alcalase, this enzyme is suitable when using acidic conditions.

3.3. Identification of peptides in hydrolysates

Peptides in the hydrolysates obtained by the three Maxipro enzymes were sequenced by RP-HPLC-ESI-Q-TOF-MS/MS. Peptides identified by database searching against *Olea europaea* organism are included in Table 4 along with parent proteins and some peptide characteristics.

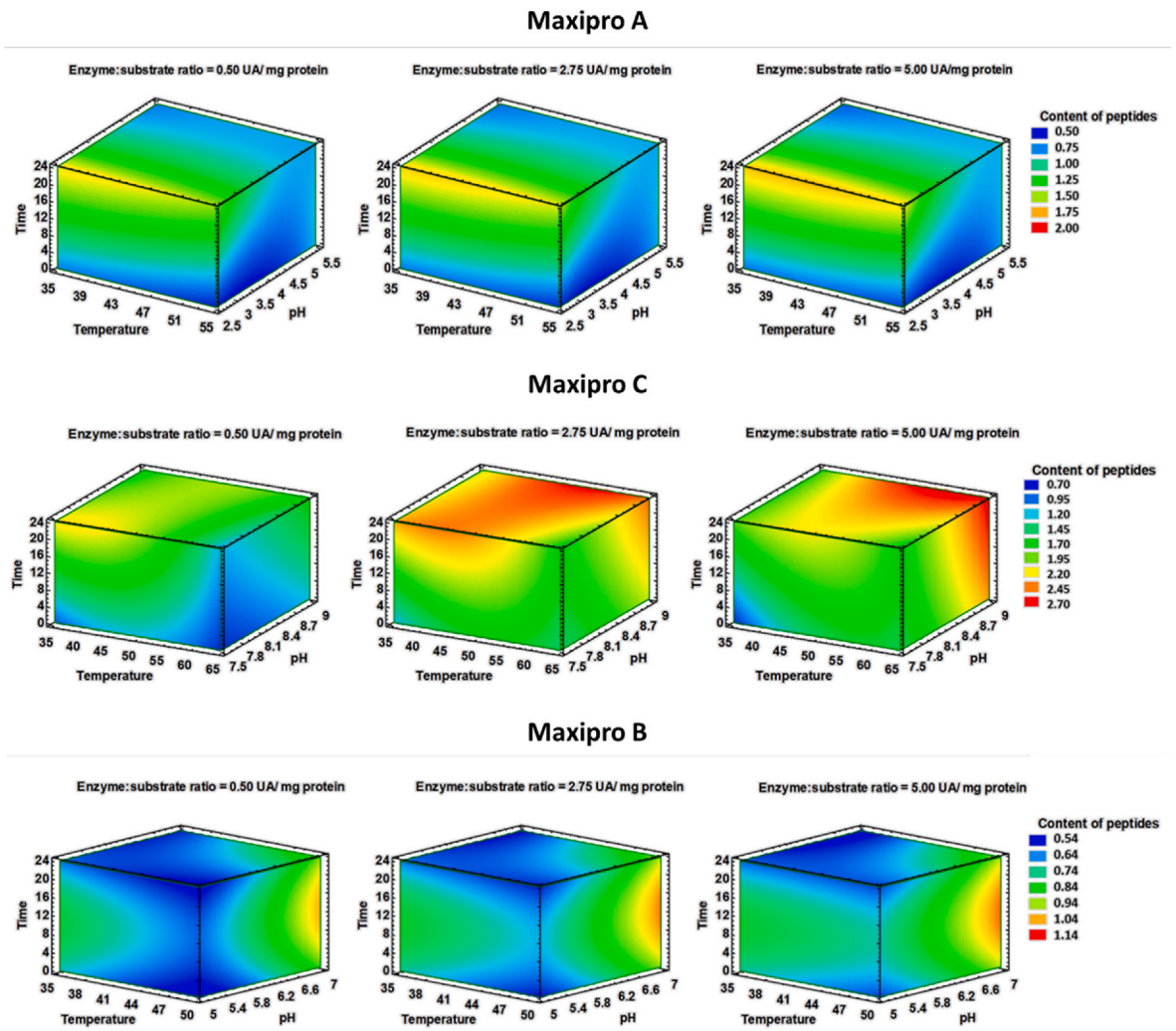

Fig. 1. 3D contour plots showing the effect of the time, temperature, and pH on the peptide content in hydrolysates obtained with Maxipro enzymes at different enzyme:substrate ratios.

Table 3
Optimal hydrolysis conditions and peptide content predicted by the mathematical model and experimentally obtained in hydrolysates.

Enzyme	Temperature (°C)	pH	Time (h)	Enzyme:substrate ratio	Peptide content (mg)*	
					Predicted	Experimental ± standard error
Maxipro A	40.3	2.5	24.0	5.0	1.72[a]	1.69 ± 0.10[a]
Maxipro C	57.8	9.0	23.9	4.2	2.77[b]	2.82 ± 0.11[b]
Maxipro B	50.0	7.0	11.9	4.8	1.07[c]	1.11 ± 0.17[c]

*Different letters means significant differences ($P < 0.05$).

Twelve peptides were identified in the hydrolysate from the Maxipro A, nine in the case of the Maxipro C, and five in the hydrolysate from Maxipro B. Hydrolysate from Maxipro C and B showed two common peptides while no common peptide was observed with the hydrolysate from Maxipro A. Peptides ranged from 5 to 12 amino acids and, in most cases, they showed good water solubility. Moreover, 67% of peptides presented acidic isoelectric points (according to Innovagen's peptide property calculator) which is a characteristic of hypocholesterolemic peptides that usually contain acidic amino acids (aspartic acid (D) and glutamic acid (E)).

Some of these peptides (AGTTPVQK, DISST, and AFLGK in the Maxipro A hydrolysate and QAGYD in the Maxipro C and B hydrolysates) were released from a protein (P24819) from the *Arabic mosaic virus*. Information related to cell location, function, and biological process of parent proteins from *Olea europaea* organism listed in Table 4 have been included in Table 5. Some of these proteins were profilins with actin binding capacity and involved in the reconstruction of cytoskeleton. One peptide (EGLYS) was released from the pollen allergen Ole e1, which is the major allergen from olive pollen. Two peptides (PKNIY and SPFPI) were part of an isoflavone reductase-like protein (phytoalexin), involved in the biosynthesis of isoflavonoids, that acts as a plant response to fungal pathogens (Cheng et al., 2015). Another peptide (SVRLN) was released from an enzyme (maturase K) involved in the excision of introns during gene expression (Barthet, Pierpont, & Tavernier, 2020) and different peptides were released from pectinesterase proteins that act in cell wall metabolism by catalysing the de-esterification of pectin.

Additional peptides were identified by *de novo* sequencing: 33 in the

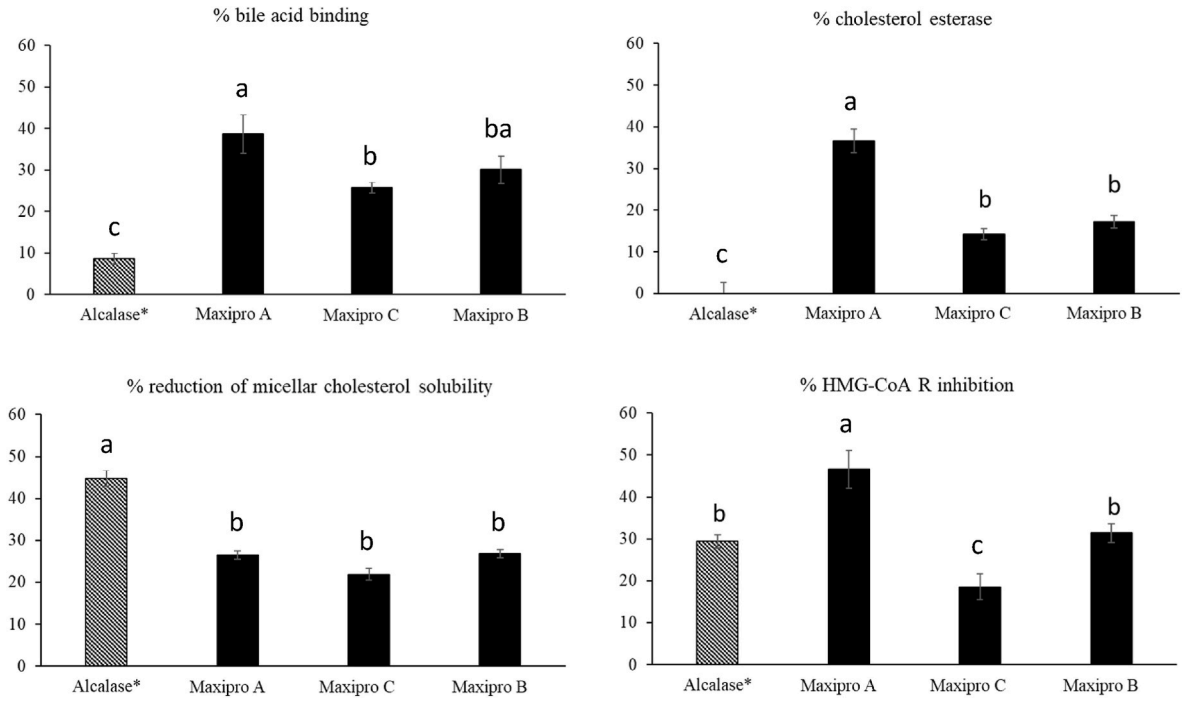

Fig. 2. Capacity of hydrolysates obtained with different enzymes, under optimal conditions, to bind bile acids, to inhibit cholesterol esterase and HMG-CoA reductase, and to reduce micellar cholesterol solubility. Different letters represent significant differences ($P \leq 0.05$). * Data from reference 8.

Table 4
Sequence, accession number of parent proteins obtained by database searching, retention time, m/z, theoretical mass, mass accuracy, water solubility, and isoelectric point of peptides identified in the hydrolysis of olive seed proteins with Maxipro enzymes.

Maxipro A							
Peptide sequence	Protein accession number	Retention time (min)	m/z	Theoretical mass (Da)	Mass accuracy (ppm)	Water solubility	Isoelectric point
AGTTPVQK	P24820|VP3_ARMV	3.44	801.4434	800.4392	−3.9	good	10.19
ALVTAE	D8VPP5|AL11A_OLEEU	27.26	603.3347	602.3275	−0.2	poor	1.00
DISST	P24819|POL2_ARMV	16.33	522.2429	521.2333	4.4	good	0.75
DVYNVEPET	C0HK96|Y0001_OLEEU	15.89	1065.4645	1064.4662	−8.4	good	0.64
EGLYS	P19963|ALL1_OLEEU	10.35	568.2574	567.2540	−6.8	good	1.01
GAVIR	A4GDS7|PROBB_OLEEU	2.21	515.3306	514.3227	1.3	good	10.84
NVEPET	C0HK96|Y0001_OLEEU	5.84	688.3119	687.3075	−4.2	good	0.82
PALVTAEG	D8VPP5|AL11A_OLEEU	27.50	757.4095	756.4017	0.7	poor	0.90
PETLR	C0HK96|Y0001_OLEEU	2.19	615.3463	614.3387	0.5	good	7.31
SFLGK	P24819|POL2_ARMV	3.07	551.3206	550.3115	3.3	good	0.96
TMDPAL	D8VPP5|AL11A_OLEEU	18.42	647.3049	646.2996	−3.1	poor	3.32
VILSLAPGNYSE	D8VPP5|AL11A_OLEEU	28.75	1262.6646	1261.6554	1.5	poor	1.00
Maxipro C							
Peptide sequence	**Protein accession number**	**Retention time (min)**	**m/z**	**Theoretical mass (Da)**	**Mass accuracy (ppm)**	**Water solubility**	**Isoelectric point**
AGAVI	A4GDQ6|PROAK_OLEEU	9.27	430.2651	429.2587	−2.2	poor	3.72
EGWIL	D8VPP5|AL11A_OLEEU	27	617.3282	616.322	−1.7	poor	0.92
EPLTPGQ	A4GFC2|PROCF_OLEEU	15.81	741.3773	740.3704	−0.6	good	0.99
IAGEPGA	A4GDR8|PROAX_OLEEU	4.02	614.3135	613.3071	−1.4	good	0.91
PKNIY	E1U332|ALL12_OLEEU	35.75	634.3585	633.3486	4.2	good	10.9
QAGIY	P24819|POL2_ARMV	13.1	551.2806	550.2751	−3.2	poor	3.36
RLGDY	A4GDQ6|PROAK_OLEEU	3.11	623.3139	622.3074	−1.3	good	6.34
SPFPI	E1U332|ALL12_OLEEU	21.01	560.3079	559.3005	0.2	poor	3.39
SVRLN	Q8M998|MATK_OLEEU	2.62	588.3464	587.3391	0.1	good	10.57
Maxipro B							
Peptide sequence	Protein accession number	Retention time (min)	m/z	Theoretical mass (Da)	Mass accuracy (ppm)	Water solubility	Isoelectric point
AEGQAKVI	B2VPR8|AL11B_OLEEU	10.47	815.4586	814.4548	−4.3	good	6.91
AGAVI	A4GDQ6|PROAK_OLEEU	9.04	430.2639	429.2587	−4.9	poor	6.72
NVEPE	C0HK96|Y0001_OLEEU	2.68	587.2668	586.2598	−0.4	good	0.85
QAGIY	P24819|POL2_ARMV	13.38	551.2803	550.2751	−3.7	poor	3.36
VPGDQ	B2VPR8|AL11B_OLEEU	7.75	515.2458	514.2387	−0.4	good	0.76

Table 5
Accession number, name, cell location, molecular function, and biological process of proteins releasing peptides in the hydrolysis of olive seed proteins with Maxipro enzymes.

Accession number	Name	Cell location	Molecular function	Biological process	Enzyme used in the hydrolysis
D8VPP5	Pectinesterase 1	Extracellular region	Peptinecterase activity	Pectin catabolic process/cell wall modification	Maxipro A, C
C0HK96	Uncharacterized				Maxipro A, B
P19963	Pollen allergen Ole e1	Extracellular region/endoplasm reticulum	–	–	Maxipro A
A4GDS7	Profilin 2	Cytoskeleton	Actin binding	–	Maxipro A
A4GDQ6	Profilin 3	Cytoskeleton	Actin binding	–	Maxipro C, B
A4GFC2	Profilin 4	Cytoskeleton	Actin binding	–	Maxipro C
A4GDR8	Profilin 2	Cytoskeleton	Actin binding	–	Maxipro C
E1U332	Isoflavone reductase-like	Cytoplasm	Oxidoreductase activity	–	Maxipro C
Q8M998	Maturase K	Chloroplasm/cytoplasm	RNA binding	mRNA and tRNA processing	Maxipro C
B2VPR8	Pectinesterase 2	Extracellular region	Peptinecterase activity	Pectin catabolic process/cell wall modification	Maxipro B

hydrolysate from Maxipro A, 57 in the hydrolysate obtained with Maxipro C, and 45 in that of the Maxipro B. The sequence of these peptides is included in Table S4 along with some characteristics. Unlike peptides identified by database searching showing mainly acidic pHs, isoelectric points of peptides identified by *de novo* were in the pH range 0–11. The hydrolysate from Maxipro A yielded peptides equally shared at all pH ranges (<4.14, from 6 to 8, and >9) while most peptides (58%) released by Maxipro C showed acidic isoelectric points, and peptides in the hydrolysate from Maxipro B were basic (42%). Some peptides were observed in, at least, two of the hydrolysates (see Table 6). Hydrolysates from Maxipro B and C showed the highest number of common peptides. Especially interesting were peptides LVTPH, LMAPH, ALMAPH, SVNDL, and VVVVPH since they were related with hypocholesterolemic activity (in bold in Table 6), according to a previous work of our research team (Prados, Plaza, et al., 2020). Additional peptides with hypocholesterolemic features were observed in the hydrolysate from Maxipro C (MDMS, NFAVVK, VFDGEVK, VPLSPT, and WVAF) and Maxipro B (VPGDQ). These peptides showed a predicted probability to be bioactive from 0.067 to 0.91 (over 1), according to PeptideRanker tool (http://distilldeep.ucd.ie). Higher values were observed for peptides WVAF (0.9130), LMAPH (0.4985), and MDMS (0.4873).

Table 6
Summary of peptides identified in olive seed hydrolysates by database searching and *de novo* sequencing.

Peptide sequences	Enzyme used in the hydrolysis
Peptides observed in multiple hydrolysates	
LENHLE, **LVTPH,** VLAE	Maxipro A, C, B
LMAPH, VVLK	Maxipro A, C
GVAAMK, TTPYYNSK	Maxipro A, B
AGAVI, **ALMAPH,** ALMSPH, ALVTPH, AVVK, DVYNPR, HQKLGNF, KVSSPL, LAGY, LLDTA, QAGIY, **SVNDL, VVVVPH,** WSMH, YLTHR	Maxipro C, B
Specific peptides	
AELVQK, AGTTPVQK, AGVAAMK, AGVAAVR, ALVTAE, AQVLK, ASRYP, AYAVK, DDLPR, DDLRPR, DISST, DLYSR, DVYNVEPET, EGLYS, ETLCT, ETLR, FEET, FHVVVPHNF, FTTAN, FTTANS, GAVIR, LDTANE, LMSPH, LPAGAVH, LTAQEPTR, MESGWSSGK, NNLAAGAAH, NVEPET, PALVTAEG, PETLR, SFLGK, SYDYNDD, TMDPAL, TNNWNQL, VAFK, VILSLAPGNYSE, VLANA, VVLQ, YAVK	Maxipro A
DDLPRFR, DLYNPR, DNVFK, EGWIL, ELLL, EPLTPGQ, FDGEVK, FFGF, FLPH, HTLY, IAGEPGA, KALM, KLPLL, LALLGL, LLPVLL, LLPVNLL, LLPVNTL, LSPL, LVVDGE, LWLY, LYNPR, **MDMS,** NDEGFVK, NDVFK, **NFAVVK,** NLEAGGLQ, NVDLE, NVPNLGQQ, PKNIY, QLSPN, RLGDY, SMPVDVL, SPFPI, SVRLN, TLPLL, TLPLLL, TNLE, VDGEGY, VFDGE, **VFDGEVK,** VLPR, VNDL, **VPLSPT,** VYLE, **WVAF,** WVEFN	Maxipro C
AEGQAKVI, AKAGGF, AVVSVN, EEQLRAM, EFDRAD, ESANEM, GAAHW, GLVLK, KGALVTPH, KLNAL, KVLAD, KYMDM, LLDTANE, LLRP, LSGLDKQ, LTPLLNYL, MDMSAE, NVDLETAK, NVEPE, NVKSL, PYYNSK, QLRAM, QLSAVK, SSSVVLK, TAKAGGF, TLVYATK, TSVLR, VFGSQ, **VPGDQ,** VYVAQ	Maxipro B

Bold peptides were related to hypocholesterolemic activity.
Underlined peptides also appeared in the hydrolysate obtained with Alcalase.

Comparing with peptides identified in the hydrolysate obtained with Alcalase (Prados, Plaza, et al., 2020), Maxipro C was the enzyme yielding a higher number of common peptides. This could be somehow expected taking into account their similarity since both enzymes work at a similar temperature and pH and come from the same organism. Other peptides in the Alcalase hydrolysate were within larger peptides detected in hydrolysates obtained with the new enzymes: peptide LVVD is within LVVDGE (Maxipro C), KLGNF is part of HQKLGNF (Maxipro C and B), MDMS is part of MDMSAE (Maxipro C), TLVY is part of TLVYATK (Maxipro B), VDLE is part of NVDLE (Maxipro C) and NVDLETAK (Maxipro B), LVTPH is part of peptides KGALVTPH and ALVTPH (Maxipro A, C, and B), and VVPH is part of peptides VVVVPH (Maxipro C and B) and FHVVVVPHNF (Maxipro A).

4. Conclusions

Three novel proteolytic enzymes (Maxipro A, Maxipro C, and Maxipro B) have demonstrated capability to release peptides with multitask hypocholesterolemic activities. Hydrolysis with Maxipro A was promoted at low pHs and long hydrolysis times, while Maxipro C required long times and enzyme:substrate ratios, and the hydrolysis with Maxipro B improved at high pHs. Maxipro A resulted in the hydrolysate with the highest capacity to bind bile acids and to inhibit cholesterol esterase and HMG-CoA R enzymes. It also produced peptides with capacity to reduce micellar cholesterol solubility. The highest number of common peptides with the hydrolysate obtained with Alcalase was detected when using Maxipro C, which works at alkaline pH and comes from the same organisms, and the least number with Maxipro A, requiring acid pH. Eleven peptides showed hypocholesterolemic features. This work demonstrates that the hydrolysis of olive seeds proteins using novel enzyme Maxipro A, working at acidic pH, releases peptides with multifunctional and higher hypocholesterolemic capacity than the obtained with Alcalase, under alkaline conditions.

CRediT authorship contribution statement

Isabel M. Prados: Formal analysis, Investigation, Methodology, Software, Validation, Visualization. **Elena Dominguez-Vega:** Conceptualization, Data curation, Resources, Supervision, Writing – review & editing. **M. Luisa Marina:** Funding acquisition, Project administration,

Resources, Writing – review & editing. **M. Concepción García:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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

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