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Original article

Acetylcholinesterase-inhibitor hydrolysates obtained from '*in vitro*' enzymatic hydrolysis of mannoproteins extracted from different strains of yeasts

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Summary *In vitro* inhibitory activity against acetylcholinesterase (AChE) of peptides obtained by enzymatic hydrolysis of mannoproteins extracted from strains of yeasts was investigated. Yeast mannoproteins were extracted from strains belonging to the genera *Brettanomyces*, *Candida*, *Pichia* and *Saccharomyces* isolated from dairy products. They were obtained by heat treatment in citrate buffer and purified by affinity chromatography with concanavalin A. Each purified extract was subsequently hydrolysed with proteolytic enzymes (trypsin, pepsin, chymotrypsin and proteinase K) applied individually or in combination, thus generating smaller peptides. Inhibitory activity of the latter against AChE was determined. The molecular weight of mannoproteins, determined by SDS-PAGE, was between 6.5 and 30 kDa. As regards AChE inhibition, a preliminary screening of all hydrolysed extracts was performed, yielding variable results with 59% maximum inhibition. Subsequently, when inhibitory concentration 50 (IC50) was determined, the extracts showed higher inhibitory activity (between 6.75 and 12.3 mg mL^{-1}). Results showed that the mannoproteins separated from yeast strains of food origin generated bioactive peptides by enzymatic hydrolysis, which can be of interest to the manufacturing of food with potential functional properties.

Keywords Acetylcholinesterase, bioactive peptides, functional food, mannoproteins, yeasts.

Introduction

Acetylcholinesterase (AChE) regulates the transmission of nerve impulses in cholinergic synapses by hydrolysis of neurotransmitter acetylcholine (ACh) into choline and acetic acid (Keane & Ryan, 1999). AChE is one of the more effective enzymes as it can hydrolyse 5000 ACh molecules/molecule of enzyme/sec (Kraut *et al.*, 2000). Alzheimer's disease (AD) is a neurodegenerative disease manifested as cognitive impairment and behavioural disorders. It is characterised in its typical form by an immediate loss of memory and other mental abilities, accordingly as nerve cells (neurons) die and different areas of the brain atrophy (Hostettmann *et al.*, 2006). This disease has no cure and is terminal within 3–7 years of diagnosis. Although initiation factors still remain to be elucidated, it is well established that AD is associated with reduced levels of ACh, which is the major neurotransmitter in the central nervous system (Singh, 2003). According to the cholinergic hypothesis, the restoration of the levels of ACh, which are gradually lost during the progress of AD, slows the loss of cognitive function (Filho *et al.*, 2006). Recent studies have shown that AChE inhibitors relieve neuropsychiatric symptoms in patients with AD and provide beneficial effects on cognitive ability by increasing levels of ACh in the synaptic region (Zarotsky *et al.*, 2003). AChE inhibition also serves as a strategy for treating other neurological disorders such as senile dementia, ataxia, myasthenia gravis and Parkinson's disease (Pulok *et al.*, 2007). In this field, research has made the greatest therapeutic advances in the treatment of AD by inhibiting AChE (Sramek *et al.*, 2002; Singh, 2003), and therefore, the search for other inhibitors of this enzyme has increased markedly. Some AChE inhibitor compounds have been found from various natural sources, mainly from plants (Murray *et al.*, 2013) as well as from animal proteins (Ahn *et al.*, 2010) or mushrooms (Patocka, 2012) or from marine photosynthetic organisms (Custódio *et al.*, 2012).

Closely related to this search, nowadays fully embrace that functional food, besides play the role of

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nutrients can provide bioactive compounds to the human body, health benefits and reduce the risk of certain diseases. Researches in the design and development of functional foods have dedicated special attention to the study of the physiological role of dietary proteins. Bioactive peptides encrypted in the structure of these proteins are usually generated *in vivo* by the action of gastrointestinal enzymes, or may also be obtained *in vitro* with specific enzymes, and/or occur during the development of biotechnological processes of certain foods. These bioactive or functional peptides are defined as inactive sequences of amino acid residues inside the precursor protein, which upon release exert specific biological activities (Meisel, 1998). Bioactive peptides may exert their effects on the cardiovascular, digestive, immune and nervous systems and among others (Korhonen & Pihlanto, 2003).

Mannoproteins are proteins bound to sugar molecules (glycoproteins), specifically mannose residues, in a proportion ranging between 50% and 90%. They are a component of the cell wall of yeasts that have been most studied in recent years. However, compounds remain little used in the food industry, despite they are readily available, inexpensive and with numerous properties. In addition, there are several food containing naturally mannoprotein from yeast wall and ingested therewith naturally, so it is a group of GRAS (generally regarded as safe) compounds (Núñez *et al*., 2006). Possible technological and industrial applications of yeast mannoproteins include control of pathogens in meat and poultry industry, increased immune response, improvements in digestion and absorption of nutrients in animals intended for human consumption, improving various wine organoleptic characteristics and of different oenological procedures. In addition, it was observed that the mannoproteins may have functional attributes for human consumption, which could contribute to increasing the quality and food safety and thus consumer welfare.

As it is necessary to find new therapeutic agents against AD, the aim of this study was to evaluate the *in vitro* inhibitory activity against AChE of peptides obtained by enzymatic hydrolysis of mannoproteins extracted from five yeast strains isolated from food products from Santa Fe and Patagonia regions. These peptides could not only provide new therapeutic agents but also prevent the onset of the disease if incorporated with functional foods containing them.

Materials and methods

Yeast strains

Saccharomyces cerevisiae DBFIQ L2, *Candida blankii* LTW7 *Candida famata* DBFIQ L17, *Brettanomyces intermedius* DBFIQ L12 and *Pichia anomala* LTW6, isolated from dairy products (cheeses, raw milk and cheese whey) of the Santa Fe and Patagonia regions, were obtained from the own collection of Cátedras de Microbiología y Biotecnología (FIQ-UNL). These strains were stored by cryopreservation at −80 °C and activated for use by successive inoculations in malt extract–yeast extract broth (YM broth) (malt extract: 3 g; yeast extract: 3 g; tryptone: 5 g; dextrose: 10 g; distilled water: 1000 mL), incubated at 30 °C for periods of 48 h, with stirring.

Mannoprotein heat extraction

The extraction of mannoproteins was performed according to the method of Peat *et al*. (1961), modified by Cameron *et al*. (1988). Each yeast strain was activated by three successive cultures in YM broth, incubating each culture at 30 °C for 48 h. The final culture was obtained by seeding 4 mL of previous culture in 400 mL of YM broth and incubating at 30 °C for 48 h with stirring. Yeast cells were collected from each culture by centrifugation at 3000 ***g*** for 15 min at room temperature and subsequently washed twice with Milli-Q water. Then, the collected cells were resuspended in a solution of 20% (v/v) of buffer 0.1 mol L^{-1} potassium citrate – 0.02 mol L^{-1} potassium metabisulphite. The pH of the slurry was adjusted to 7.0 and the mixture was subjected to heat treatment in an autoclave for 2 h at 121 °C. Then, the resulting suspension was centrifuged at 5000 ***g*** for 10 min at room temperature. The supernatant was mixed with three volumes of 95% ethanol containing 1% (v/v) acetic acid and allowed to stand overnight at 4 °C. The precipitate was collected by centrifugation at 10 000 ***g*** for 10 min at 5 °C. The extracts obtained were preserved by lyophilisation and stored at 4 °C until use.

Determination of protein concentration

The concentration of protein in the extracts obtained was determined by the bicinchoninic acid assay (Fujimoto *et al*., 1985). Bovine serum albumin was used as a standard.

Mannoproteins purification by affinity chromatography

The purification of each extract was performed by affinity chromatography using concanavalin A (Con A)-Sepharose 4B. Mannoproteins were eluted with 0.1 mol L^{-1} α-methyl D-mannopyranoside in equilibrium buffer (0.01 mol L^{-1} Tris-HCl (pH 7.5) containing 0.5 mol L^{-1} NaCl, 1 mmol L^{-1} $CaCl_2$ and 1 mmol L^{-1} $MnCl_2$) and were detected by measuring absorbance at 280 nm employed spectrophotometer Metrolab, Model 1700. Each mannoprotein extract was dialysed using membranes with 1-kDa cut-off

and was subsequently lyophilised and stored at −4 °C (Ahmed, 2004). In this chromatography, a glass column (length: 180 mm, diameter: 13 mm), a peristaltic pump Econo Gradient Pump and fraction collector Model 2110 (the three of them Bio-Rad Laboratories, Inc. Hercules, CA, USA) were used.

Enzymatic digestion of mannoproteins

Enzymatic hydrolysis of each mannoprotein extract was first conducted with trypsin (E.C.: 3.4.21.4), pepsin (E.C.: 3.4.23.1) or proteinase K (E.C.: 3.4.21.64). In addition, each of these hydrolysed extracts was treated with chymotrypsin (E.C.: 3.4.21.1). This digestion was carried out at 37 °C for 24 h for all enzymes, except for proteinase K, which was performed for 18 h. In all tests, a protein–enzyme ratio 50:1 (w/w) was used. For hydrolysis with trypsin, chymotrypsin and proteinase K, buffer 30 mmol L^{-1} Tris-HCl, pH 8.0, was used. For hydrolysis with pepsin, an aqueous solution of acetic acid (5% (v/v)), pH 2.0, was used. The enzymatic reaction was stopped by heating at 100 °C for 10 min. All enzymes were supplied by Sigma Chemical Co. (St. Louis, MO, USA).

Analysis by SDS-PAGE of mannoproteins and their hydrolysates

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on discontinuous polyacrylamide gels (stacking gel: 4%, separation gel: 16%) under reducing conditions, according to the method of Laemmli (1970), using glycine as trailing ion. Protein bands were visualised by silver staining (Blum *et al*., 1987).

To perform the assays, concentrations of 1, 5 and 10 mg mL^{-1} of mannoproteins and their hydrolysates were used.

A kit of standard proteins (low-range molecular weight 6.5–66 kDa; Sigma) was used as molecular weight markers.

Determination of degree of hydrolysis

Mannoproteins hydrolysed extracts were prepared at a concentration of 1 mg mL^{-1}, using for assays 1/10 and 1/20 dilutions thereof. The degree of hydrolysis was determined by reaction of the free amino groups with o-phthalaldehyde (OPA) in the presence of dithiothreitol (DTT), forming a coloured compound that is detected by absorbance at 340 nm, following the procedure described by Nielsen *et al*. (2001). The total number of free amino was determined previously on each hydrolysed sample by 100% with 6 N HCl for 20 h at 105 °C.

Analysis by MALDI-TOF

For the acquisition of the MALDI-TOF spectra, the samples were previously desalted by size exclusion chromatography using Sephadex G-10 (Sigma) and subsequently lyophilised. The mass range investigated was up to 6 kDa.

Mass spectrometric data were obtained using a MALDI-TOF-TOF spectrometer, Ultraflex II (Bruker, Billerica, MA, USA), in the mass spectrometry facility CEQUIBIEM, Argentina.

Acetylcholinesterase inhibitory activity

The AChE inhibitory activity was determined using the spectrophotometric method described by Ellman *et al*. (1961); 100 μL of each sample was incubated with 7 μL of a solution of human red blood cells group '0' RH(+), for 30 min at room temperature. Subsequently, 450 μL of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and 450 μL of acetylthiocholine were added, proceeding immediately to the spectrophotometric reading at 405 nm and obtaining the enzymatic initial rate (ΔAbsorbance/30 s). A solution of galantamine 200 μmol L^{-1} was used as positive control of the inhibition and phosphate buffer pH 7.7 as enzyme basal activity control.

Calculation:

$$\text{Enzymatic activity}\,(\text{U L}^{-1}) : (\Delta\text{Absorbance}/30\,\text{s}) \times \text{Factor}$$

$$\%\ \text{Enzymatic activity}\,(\%\ \text{EA}) : (\text{EAM}/\text{BEA}) \times 100$$

$$\%\ \text{Inhibition} : 100 - \%\ \text{EA}$$

EAM: Enzymatic activity of the sample; BEA: basal activity enzymatic; Factor: 286764.

Factor: [10^6 × final assay volume (l) × dilution factor)/(ε × optical path (cm) × sample volume (l)].

ε: molar extinction coefficient: 13 600 l mol^{-1} cm^{-1}.

On the other hand, a microplate assay using the spectrophotometric method described by Ellman *et al*. (1961) with some modifications (López *et al*., 2002) (described in the next section) was also employed, for comparative purposes with the previously described.

Determination of inhibitory concentration 50

A microplate assay using the spectrophotometric method described by Ellman *et al*. (1961) with some modifications (López *et al*., 2002) was also employed for the determination of the Inhibitory concentration 50 (IC50) of the most active extracts. In this assay, AChE (Sigma) was used, and 50 μL of each sample was incubated with 50 μL of AChE (0.25 U mL^{-1}) in a 96-well microplate for 30 min at room temperature.

Subsequently, 100 μL of the substrate solution (DTNB 0.2 mmol L^{-1}, acetylthiocholine iodide 0.24 mmol L^{-1} and Na_2HPO_4 0.04 mmol L^{-1}, pH 7.5) was added. The absorbance was read in an ELISA detector at 405 nm after 5 min. As a positive inhibition control, galantamine solution 200 μmol L^{-1} was used, and as basal activity control of the enzyme, phosphate buffer pH 7.5 was used. The inhibition activity is expressed as the concentration of hydrolysate needed to inhibit 50% of the original enzyme activity (IC50). Concentration–activity curves were generated for concentration of sample (abscissa) vs. % AChE inhibitory activity (ordinate). The ranges of concentrations of the mannoproteins extracted use in this assay were 0.5 and 80 mg mL^{-1}. The IC50 value was obtained by the parameters of the fitted function using Origin 8 program.

$$Y = \min + (\max - \min)/1 + 10^{(\log \mathrm{IC50} - \mathrm{x}) * \mathrm{hillslope}}$$

In this equation, Y represents the AChE inhibitory activity (%) and x the logarithm of the concentration of inhibitor (mg mL^{-1}). Parameter min equals the baseline of 100% inhibition and max the plateau of 100% activity. Parameter IC50 gives the transition centre. The hill slope determines the slope of the curve at the transition centre.

Statistical analysis

All experiments were performed in triplicate. The values were reported as mean ± standard deviation. Statistically significant differences among means of experimental design were studied by analysis of variance (ANOVA) at $P \leq 0.05$.

Results

Mannoproteins contained in the crude extracts were separated by Con A lectin affinity chromatography. Figure 1 shows the chromatographic pattern of Con A purification of mannoprotein extracts. When eluting after methyl α-D-mannopyranoside treatment, only one or two peaks indicative of mannoproteins in each extract were detected between tubes 12 and 26.

Peaks between tubes 0 and 10 correspond to the rest of the crude extract, which was not stalled on the affinity column. The resulting mannoprotein extracts were dialysed, lyophilised and stored at 4 °C for later use.

Table 1 indicates the amount of crude and separated mannoprotein in the different steps for each yeast strain under study. It is seen that thermal extraction procedure allowed to isolate mannoproteins from the cell walls of all yeast strains studied. The percent-

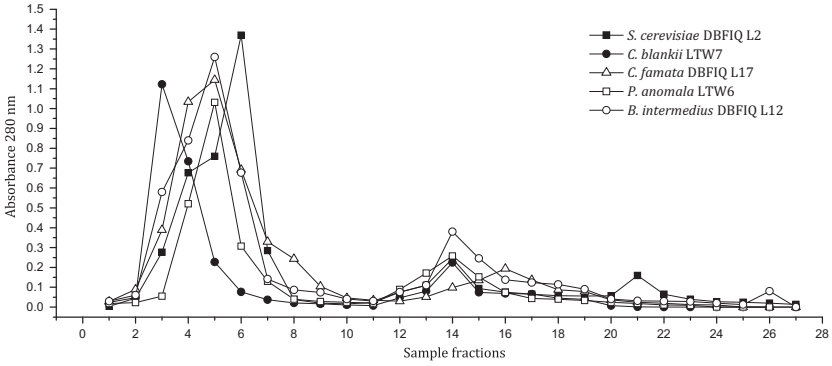

Figure 1 Affinity chromatography elution profiles of each mannoproteins extract. Chromatographic pattern of mannoprotein extracts using Con A after treatment with methyl α-D-mannopyranoside. The mannoprotein peaks eluted between fractions 12 and 26.

Table 1 Yield and characterisation of mannoproteins extracted from five strains of yeast

Yeast	Wet weight (cells) (g)	Wet weight of crude extract (obtained after heat treatment) (g)	Dry weight of crude extract (lyophilised) (mg)	Amount of protein (mg total protein per g of crude lyophilised extract)	% mannoproteins [g mannoproteins/ 100 g cells (wet weight)]
Pichia anomala LTW6	8.3	1.9	499.5	330	3.41
Candida blankii LTW7	8	1.2	627.3	390	5.30
Candida famata DBFIQ L17	7.8	1.62	494.8	410	3.52
Saccharomyces cerevisiae DBFIQ L2	9.3	2.2	514.8	360	3.55
Brettanomyces intermedius DBFIQ L12	2.8	0.581	218.5	245	3.45

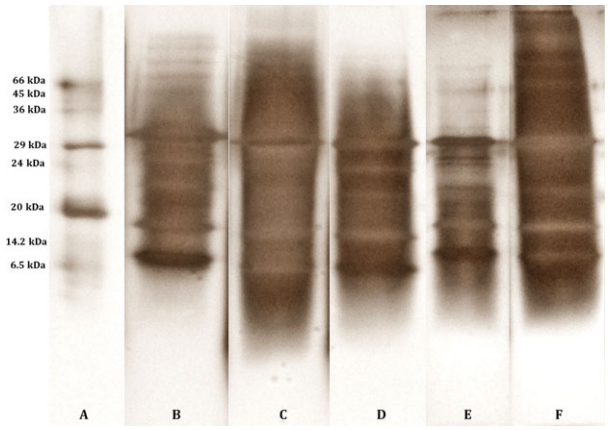

Figure 2 SDS-PAGE of each of the FIVE extracts separated by affinity chromatography. (A): MW markers, (B): mannoprotein crude extract of *P. anomala* LTW6 purified by Con A, (C): mannoprotein crude extract of *C. blankii* LTW7 purified by Con A, (D): mannoprotein crude extract of *S. cerevisiae* DBFIQ L2 purified by Con A. (E): mannoprotein crude extract of *B. intermedius* DBFIQ L12 purified by Con A, (F): mannoprotein crude extract of *C. famata* DBFIQ L17 purified by Con A.

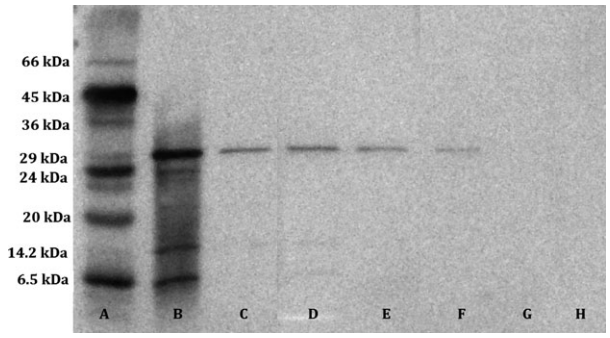

Figure 3 SDS-PAGE of *Saccharomyces cerevisiae* DBFIQ L2 mannoproteins hydrolysated with different proteases. (A): MW markers, (B): mannoprotein extract of *S. cerevisiae* DBFIQ L2 purified by Con A, (C): extract hydrolysed with trypsin, (D): extract hydrolysed with proteinase K, (E): extract hydrolysed with pepsin, (F): extract hydrolysed with trypsin/chymotrypsin, (G): extract hydrolysed with pepsin/chymotrypsin (H): extract hydrolysed with proteinase K/chymotrypsin.

age of mannoproteins obtained per gram of cells (wet weight) was similar in all cases except for *C. blankii* LTW7.

Figure 2 shows the results obtained when analysing mannoprotein fractions by SDS-PAGE. Bands of different molecular weights (MWs) were observed on the street corresponding to the purified mannoproteins. These bands indicate not only that heat extraction produces heterogeneous protein fractions, but also that the MWs of the purified mannoproteins were similar in all cases (between 6.5 and 30 kDa).

Hydrolysis of each mannoprotein extract with proteolytic enzymes resulted in the decrease of the protein bands obtained by SDS-PAGE. Figure 3 shows enzymatic hydrolysis treatments of the extract obtained from *S. cerevisiae* DBFIQ L2 strain. A marked reduction of mannoprotein bands after treatment with trypsin, pepsin, proteinase K and trypsin/chymotrypsin and their complete disappearance after the treatment with pepsin/chymotrypsin and proteinase K/chymotrypsin were detected. For the remaining mannoprotein extracts, similar results were observed (data not shown), which indirectly would indicate the formation of a large number and variety of peptides, whose biological activity is important to study.

As results obtained with different concentrations of mannoproteins and their hydrolysates (1, 5 and 10 mg mL^{-1}) were coincident, Figs 2 and 3 show only those results corresponding to a concentration of 1 mg mL^{-1}.

The extent of proteolysis was quantified as the degree of hydrolysis (DH) using the OPA method and refers to the percentage of peptide bonds cleaved. Table 2 indicates the degree of hydrolysis of all mannoprotein extracts treated with trypsin, pepsin, proteinase K and chymotrypsin. It can be appreciated that the doubly hydrolysed extracts (chymotrypsin plus other proteolytic enzyme) showed higher degree of hydrolysis, with a maximum value of 62.4%. Correlating these data with those shown in Fig. 3, which shows the results of SDS-PAGE for the hydrolysed extract of *S. cerevisiae* DBFIQ L2, the formation of peptides with a MW <6.5 kDa can be inferred.

Analysis by MALDI-TOF mass spectrometry (data not shown) confirmed that the molecular mass of the peptides present in the hydrolysed extracts of *S. cerevisiae* DBFIQ L2 is in the range of 600 to 5800 Da. This corroborates that the molecular weight of the peptides present in each hydrolysate is <6.5 kDa.

Based on the previously detailed results, it was considered important to study the potential biological activity of the peptides resulting from the hydrolysis of mannoproteins under study, investigation of their inhibitory activity against AChE being of significant importance. As a first step, a preliminary screening of the inhibitory activity against AChE enzyme of all hydrolysates of mannoproteins was performed, following the spectrophotometric method described by Ellman *et al.* (1961). The AChE inhibitory activity (%) of the hydrolysed extracts at the three concentrations tested (5, 2.5 and 1.25 mg mL^{-1}) is shown in Table 3.

The inhibitory activity against AChE of the different extracts was classified as strong (>50% inhibition), moderate (30–50% inhibition), low (<30% inhibition) or null (<5% inhibition), as suggested by Vinutha *et al.* (2007). Of the extracts hydrolysed with trypsin, only that of *C. blankii* LTW7 presented a moderate

Table 2 Determination of degree of hydrolysis of different mannoproteins subjected to different enzymatic treatments

	Degree of hydrolysis (%)					
Samples	**Trypsin**	**Pepsin**	**Proteinase K**	**Trypsin/chymotrypsin**	**Pepsin/chymotrypsin**	**Proteinase K/chymotrypsin**
P. anomala LTW6	$^{b}9.3 \pm 3.1$	$^{a}10.1 \pm 0.6$	$^{a}17.1 \pm 2.3$	$^{a}62.4 \pm 8.3$	$^{a}60.0 \pm 6.4$	$^{a}61.4 \pm 5.7$
C. blankii LTW7	$^{a}17.0 \pm 3.0$	$^{a}12.7 \pm 4.0$	$^{b}5.2 \pm 1.0$	$^{a}60.0 \pm 5.0$	$^{a}59.0 \pm 9.2$	$^{a}55.0 \pm 3.4$
S. cerevisiae DBFIQ L2	$^{a,b}14.2 \pm 1.0$	$^{a}14.5 \pm 0.8$	$^{a,b}11.0 \pm 2.5$	$^{b}35.8 \pm 5.4$	$^{a}48.7 \pm 9.0$	$^{a}50.0 \pm 7.0$
B. intermedius DBFIQ L12	$^{c}5.5 \pm 0.6$	$^{a}10.4 \pm 1.2$	$^{a}11.7 \pm 4.0$	$^{b}28.0 \pm 2.8$	$^{b}23.1 \pm 1.6$	$^{b}26.7 \pm 6.0$
C. famata DBFIQ L17	$^{a,b}14.5 \pm 0.4$	$^{b}4.0 \pm 0.4$	$^{a}12.2 \pm 0.6$	$^{b}25.7 \pm 3.0$	$^{b}20.1 \pm 1.0$	$^{b}24.2 \pm 5.4$

Results represent means ± SD of three independent experiments. The values with different superscripts indicate significant difference within the same column ($P < 0.05$).

Table 3 Screening of inhibitory activity against AChE of all mannoproteins hydrolysates

AChE inhibition (%)						
	Enzymatic treatments					
	Extracts treated with trypsin			**Extracts treated with trypsin + chymotrypsin**		
Yeast extract	**5 mg mL^{-1}**	**2.5 mg mL^{-1}**	**1.25 mg mL^{-1}**	**5 mg mL^{-1}**	**2.5 mg mL^{-1}**	**1.25 mg mL^{-1}**
P. anomala LTW6	N.I.	N.I.	N.I.	$^{b}16.3 \pm 2.2$	$^{a}15.6 \pm 3.8$	$^{a}18.4 \pm 3.8$
C. blankii LTW7	$^{a}42.6 \pm 6.0$	N.I.	$^{a}9.0 \pm 2.5$	$^{a}39.3 \pm 3.2$	$^{a}11.2 \pm 4.1$	$^{b}8.6 \pm 4$
S. cerevisiae DBFIQ L2	$^{b}8.5 \pm 1.2$	$^{a}11.8 \pm 3.3$	$^{b}16.0 \pm 1.4$	N.I.	$^{a}13.3 \pm 2.8$	N.I.
B. intermedius DBFIQ L12	N.I.	N.I.	N.I.	N.I.	N.I.	$^{b}1.8 \pm 0.7$
C. famata DBFIQ L17	N.I.	$^{a}4.3 \pm 3.8$	$^{c}1.1 \pm 0.4$	$^{a}33.8 \pm 3.7$	$^{a}13.8 \pm 2.4$	$^{a}23.2 \pm 1.7$
	Extracts treated with pepsin			**Extracts treated with pepsin + chymotrypsin**		
P. anomala LTW6	N.I.	N.I.	$^{a,b}6.9 \pm 2.2$	$^{a}21.5 \pm 3.2$	$^{a}29.2 \pm 4.4$	$^{a}41.1 \pm 5.7$
C. blankii LTW7	$^{a}7.2 \pm 5.7$	N.I.	$^{a}12.1 \pm 2.6$	$^{b}59 \pm 4.2$	$^{a}31.6 \pm 3.8$	$^{a}46.6 \pm 4.2$
S. cerevisiae DBFIQ L2	$^{a}7.6 \pm 3.6$	N.I.	$^{a}9.6 \pm 3.5$	$^{c}46.3 \pm 2.2$	$^{b}46.4 \pm 3.4$	$^{a}38.7 \pm 4.3$
B. intermedius DBFIQ L12	$^{b}30.6 \pm 3.0$	N.I.	$^{b}0.1 \pm 0.1$	$^{d}1.7 \pm 1.7$	$^{d}14.8 \pm 0.5$	$^{b}14.9 \pm 2.5$
C. famata DBFIQ L17	N.I.	N.I.	N.I.	$^{e}34.6 \pm 5.0$	$^{a}30.9 \pm 1.0$	$^{b}19.3 \pm 3.4$
	Extracts treated with proteinase K			**Extracts treated with proteinase K + chymotrypsin**		
P. anomala LTW6	N.I.	N.I.	N.I.	$^{a}21.0 \pm 4.2$	$^{a}16.3 \pm 5.1$	$^{a}19.4 \pm 1.3$
C. blankii LTW7	N.I.	N.I.	$^{a}5.3 \pm 1.3$	$^{b}1.7 \pm 0.7$	$^{a,b}8.3 \pm 0.8$	$^{a,b}13.1 \pm 5.0$
S. cerevisiae DBFIQ L2	$^{a}54.4 \pm 3.1$	37.1 ± 3.4	$^{b}18.5 \pm 6.0$	$^{c}32.1 \pm 4.5$	$^{b}1.1 \pm 0.4$	$^{b}6.7 \pm 2.5$
B. intermedius DBFIQ L12	$^{b}7.5 \pm 1.0$	N.I.	N.I.	$^{b}5.8 \pm 3.0$	$^{a}9.5 \pm 3.7$	$^{a}19.2 \pm 2.0$
C. famata DBFIQ L17	$^{c}37.3 \pm 3.9$	N.I.	$^{a,b}9.8 \pm 2.0$	N.I.	$^{a,b}9.0 \pm 2.5$	$^{b}9.8 \pm 4.0$

N.I.: Not Inhibit. Positive control: galantamine 200 mmol L^{-1}, inhibitory activity against AChE: 94.04 ± 4.5%. Results represent means ± SD of three independent experiments. The values with different superscripts indicate significant difference within the same concentration ($P < 0.05$).

activity with an inhibition value of 42.6% at a concentration of 5 mg mL^{-1}. The remaining extracts showed slight or no activity against AChE. Besides, when this same extract was hydrolysed with trypsin/chymotrypsin, the activity was slightly lower (39.3%). Of the remaining extracts hydrolysed with both trypsin and chymotrypsin, only the one corresponding to *C. famata* DBFIQ L17 strain showed moderate activity (33.8%) at a concentration of 5 mg mL^{-1}, whereas no activity was detected when it was hydrolysed only with trypsin.

With regard to extracts hydrolysed with pepsin, only that corresponding to *B. intermedius* DBFIQ L12 showed a moderate inhibitory activity against AChE, with an inhibition value of 31% at a concentration of 5 mg mL^{-1}. However, the inhibition value of this same extract hydrolysed with pepsin/chymotrypsin decreased to 2% at the same concentration. The remaining extracts showed higher activity when doubly hydrolysed with pepsin/chymotrypsin than when they were hydrolysed only with pepsin. It can be appreciated that the extract of *S. cerevisiae* DBFIQ L2 showed the highest

activity and with similar values through the range of concentrations tested (46.3%, 46.4% and 38.7% at 5, 2.5 and 1.25 mg mL^{-1}, respectively). On the other hand, the activity of the extract of some strains (*P. anomala* LTW6, *S. cerevisiae* DBFIQ L2, *B. intermedius* DBFIQ L12, *C. blankii* LTW7) increased as concentration decreased, which could be due to solubility or aggregation problems of the peptides present in this extract. The extract obtained from *C. blankii* LTW7 showed a variable inhibitory activity with a high value at a concentration of 5 mg mL^{-1} (59% inhibition), decreasing to 31.6% at a concentration of 2.5 mg mL^{-1} and increasing to 46.6% at the lowest concentration tested (1.25 mg mL^{-1}). The extract of *C. famata* DBFIQ L17, on the other hand, presented inhibition values slightly higher than 30% at concentrations of 5 and 2.5 mg mL^{-1}, when it was subjected to double enzyme treatment, showing, in turn, null activity after treatment with pepsin alone.

With respect to the extracts hydrolysed with proteinase K, significant inhibition values against AChE were found with the extract of *S. cerevisiae* DBFIQ L2 (54.5% and 37.1% at 5 and 2.5 mg mL^{-1}, respectively) and *C. famata* DBFIQ L17 (37.3% at 5 mg mL^{-1}). When these extracts were doubly hydrolysed with proteinase K and chymotrypsin, the inhibitory activity decreased significantly. No significant inhibitory activity was found in the remaining extracts.

Results obtained using the microplate assays were practically coincident with those previously described (data not shown). For this reason, and as it is more reproducible than the other method tested, microplate assay was chosen for IC50 determination.

In this way, the extracts with higher inhibitory activity against AChE were selected, and their IC50 determined, using commercial AChE (Sigma) and a micromethod (with 96-well microplates). Table 4 shows the obtained results. As seen in this table, the IC50 values ranged from 6.75 to 12.3 mg mL^{-1}, the extract of *Saccharomyces cerevisiae* DBFIQ L2 hydrolysed with pepsin and chymotrypsin having the lowest IC50 value against AChE.

Table 4 Values of IC50 of the inhibitory activity against AChE

Yeast extract	Proteolytic enzyme	IC50 (mg mL^{-1})
Candida blankii LTW7	Trypsin	12.3 ± 0.3
	Trypsin/chymotrypsin	14.4 ± 0.8
	Pepsin/chymotrypsin	11.2 ± 0.6
Saccharomyces cerevisiae DBFIQ L2	Proteinase K	13 ± 0.4
	Pepsin/chymotrypsin	6.8 ± 0.55
Pichia anomala LTW6	Pepsin/chymotrypsin	7.7 ± 0.3

Positive control: galantamine 200 mmol L^{-1}, inhibitory activity against AChE: 91.5 ± 2.7%.

Discussion

When comparing the results obtained in our thermal extraction of mannoproteins with data reported by Cameron *et al.* (1988), lower percentages of these compounds were generally observed. They detected mannoprotein percentages between 2.9% and 18% for the different yeast strains studied.

Yeast extracts are currently obtained by enzymatic or thermal processes, or by a combination of both methods. The type of method used can have a great influence on the characteristics of the obtained mannoprotein fraction (Núñez *et al.*, 2006). Cameron *et al.* (1988) found similar results in the composition of the extracts obtained by both enzymatic treatment (with zymolase enzyme) and heat treatment. Dupin *et al.* (2000a,b) evaluated different extraction methods and found that the most effective treatment for the release of mannoproteins was performed with zymolase enzyme.

In more recent studies, both methods of extraction were applied, differences being observed in the composition of polysaccharides and proteins. Thus, the enzymatic extract of mannoproteins had a greater proportion of polysaccharides than the heat extract, which, on the other hand, showed higher protein content (about threefold). Mannose was the main sugar found in glycoproteins of both extracts, confirming that, whatever the method used, mannoproteins were the major components. It was also noted that heat extraction produced more heterogeneous protein fractions than the enzymatic method (Núñez *et al.*, 2006; Gañan *et al.*, 2009).

With respect to the methodology applied for isolation of mannoproteins from crude extracts, lectins are frequently used to purify glycoproteins from biological extracts. Unlike the conventional purification procedures (e.g. ion exchange chromatography or gel filtration), which exploit the general physical properties of glycoproteins, lectins recognise specific three-dimensional structures created by a cluster of sugar residues. Conventional purification procedures are usually tested before application of the lectin affinity chromatography (Freeze, 2001). Con A has been used in procedures for affinity chromatography purification of different yeast mannoproteins (So & Goldstein, 1968; Gonçalves *et al.*, 2002; Huang *et al.*, 2002; Núñez *et al.*, 2006; Gañan *et al.*, 2009), because of its specificity for α-D-mannose and α-D-glucose residues and because the binding of soluble glycoproteins in the gel can be easily reversed by adding a low molecular weight sugar or sugar derivatives (So & Goldstein, 1968).

Comparing the results obtained in this research on SDS-PAGE chromatography for purified mannoprotein extracts with those of other authors, Gañan *et al.* (2009)

works proved to be of utmost importance as they detected bands of similar MWs in a heat-extracted sample obtained from a strain of *Candida jejuni*.

Currently, AChE inhibitors are widely used in patients with Alzheimer's disease to inhibit the hydrolysis of ACh, thereby activating the central cholinergic system and alleviating cognitive deficits (Devomoy *et al*., 2003). However, some of these chemically synthetised inhibitors appear to be giving rise to severe side effects such as nausea, vomiting, bradycardia, anorexia and sweating (Giacobini, 2004). Therefore, research on naturally occurring products without side effects has become a priority. Several studies were developed to generate bioactive peptides by enzymatic hydrolysis from food proteins. A number of enzymatic hydrolysates are widely applied to improve and enhance the nutritional and functional properties of proteins. Furthermore, the use of different enzymes with different mechanisms of hydrolysis allows obtaining bioactive peptides with different properties (Ahn *et al*., 2010). Bioactive peptides have been isolated and characterised from various sources of dietary protein, including milk and milk products, eggs, fish, oysters, cereals (rice, wheat, barley and maize), soya bean and radish seeds, among others. Within the identified bioactive functions, antihypertensive properties (angiotensin-converting enzyme inhibitors), antioxidants, anticancer, immunomodulatory and opioid agonists and antagonists can be mentioned (Wang & de Mejia, 2005).

Nowadays, very few studies have considered inhibition of the enzyme acetylcholinesterase by enzymatic hydrolysates of food proteins. Ahn *et al*. (2010) found good results studying the *in vitro* inhibitory activity against AChE of different enzymatic protein hydrolysates obtained from tuna liver. All fractionated tuna liver protein hydrolysates inhibited AChE activity, which was affected by enzymes and MW of tuna liver protein hydrolysates. In all hydrolysates produced by different proteolytic enzymes, the inhibition activities of higher MW fractions were higher than those of lower MW fractions.

No previous research on peptides derived from yeast mannoproteins has so far been found in the literature. Therefore, and to our knowledge, this would be one of the first scientific works devoted to this specific issue and could be an original contribution to this area with the potential use in the field of new therapeutic agents and functional foods.

The objective of this research has been exclusively to study the *in vitro* inhibition of AChE.

While *in vivo* studies are necessary to determine with certainty the beneficial effect of incorporating hydrolysates into functional foods, it is, however, essential to carry out prior *in vitro* studies to detect hydrolysates of mannoproteins with AChE inhibitory activity.

Conclusion

Given the complexity of AD, new therapeutic approaches to treat this disease and other neurological disorders include finding bioactive compounds that can act on multiple targets.

Results of this work have demonstrated that a large amount and variety of peptides with anti-AChE activity can be obtained from yeast mannoproteins. Some of these should be considered for future studies regarding Alzheimer's disease to prove their potential suitability for use as functional food additives and/or therapeutic agents.

This affirmation becomes particularly important if one takes into account that many tons of yeast are annually discarded as a by-product of different industries, including wine and beer production, thus being commercially unprofitable and mainly intended for animal feed due to its low cost and its high content of essential amino acids (Gañan *et al*., 2007).

Inhibition of AChE is also considered as a promising approach for both the treatment of AD and potential therapeutic applications in Parkinson's disease, ageing and myasthenia gravis, as well as in preventive medicine, through the development of functional foods.

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