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Increasing the angiotensin converting enzyme inhibitory activity of goat milk hydrolysates by cross-flow filtration through ceramic membranes

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ABSTRACT

In this work, goat milk proteins were hydrolyzed by different treatments consisting sequential or simultaneous combinations of subtilisin and trypsin. The hydrolysate showing the highest angiotensin converting enzyme (ACE) inhibitory activity was submitted to ultrafiltration in the concentration mode through tubular ceramic membranes of 50 and 15 kDa. The time evolution of the permeate flux was fitted to several models in order to identify the most feasible fouling mechanisms. The retentates and the permeates obtained after reaching a concentration factor of 3 were tested for their ACE inhibitory activity. Good enhancements were determined in the case of the permeates. Finally, the cleaning of the membrane with sodium hydroxide was successfully assayed.

Keywords: Goat milk; Protein hydrolysis; ACE inhibitory activity; Ceramic membrane

1. Introduction

The dairy industry has used membrane processing since the late 1960s to clarify, concentrate, and fractionate a variety of dairy products [1]. For instance, membrane technology allowed obtaining valuable proteins of commercial usage from whey, a byproduct of cheese production. Traditionally, membranes were used for size-based separations with relatively low-resolution requirements. Recent improvements in membrane selectivity and module design have increased the use of membranes for the isolation or purification of compounds of interest in biotechnology. Examples of these applications include the purification of antibodies, recovery of inmunoglobulins, DNA extraction, or production of isolated compounds such as lactic acid or BSA, among others [2].

An application of interest is the fractionation of protein hydrolysates to obtain peptide fractions with biological activity. The hydrolysis of proteins releases peptides (i.e. fragments of protein with a given amino acid sequence) to the medium. Some specific sequences, which are inactive inside the protein matrix, can display some biological activity depending on their amino acid sequence, molecular weight (MW), hydrophobicity, charge, and acid-basic character [3]. To this regard,

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many works have isolated active fractions from milk protein hydrolysates exhibiting a variety of biological activities, such as antioxidant, antihypertensive, antimicrobial, or immunomodulatory, among others [4].

The antihypertensive activity of a given peptide is based on its ability to inhibit the activity of the angiotensin converting enzyme (ACE). This enzyme naturally occurs in bloodstream and human tissues, and catalyses the conversion of the inactive peptide angiotensin I (DRVYIHPFHL) into angiotensin II (DRVYIHPF), which is a strong vasoconstrictor. Further, ACE degrades bradykinine (RPPGFSPFR), which loses its vasodilator activity [5]. Therefore, peptides able to inhibit the activity of ACE allow the reduction of blood pressure.

ACE inhibitory activity has been extensively studied on enzymatic hydrolysates from animal or vegetal origin [3,6]. More specifically, ACE inhibitors have been mainly identified in bovine milk, while few studies deal with other sources such as yak milk [7] or goat milk [8,9]. The enzymatic treatment, in terms of enzymes, reaction parameters, and degree of hydrolysis is crucial to determine the presence of active peptides in the hydrolysate. To this regard, a wide range of proteases such as subtilisin, trypsin, thermolysin, or pepsin has been employed to produce milk protein hydrolysates with ACE inhibitory activity [5]. A reduced number of studies have demonstrated the efficiency of enzyme combinations, employed successively or simultaneously, to obtain protein hydrolysates. For instance, Pihlanto-Leppälä et al. [10] employed combinations of trypsin, chymotrypsin, and pepsin at different sequence and reaction times to obtain milk-derived protein hydrolysates displaying ACE inhibition. Wang et al. [11] tested *in vitro* ACE inhibition of whey protein isolates produced with different enzyme combinations (alcalase-neutrase and trypsin-neutrase).

Membrane separation processes can be employed to isolate peptide fractions, some of them enriched in the active peptides. Furthermore, membrane ultrafiltration is able to remove small peptides responsible for the appearance of bitterness in whey and casein protein hydrolysates [12,13]. So far, most of the studies dealing with the fractionation of hydrolysates with biological activity employ membranes of polymeric materials such as polysulphone or polyamide. Several authors filter the crude hydrolysate through a series of ultrafiltration membranes of decreasing MW cut-off, with the aim of identifying peptides from the most active fractions [1,14,15]. Similarly, the studies dealing with large scale hydrolysates filtrations also used polymeric membranes [16,17].

Although they are rarely reported in literature for the fractionation of hydrolysates, ceramic membranes present advantages over organic ones when it comes to their implementation in industrial scenarios. They bear high temperatures (up to 350˚C) and harsh chemical conditions (pH 1–13, oxidant agents), being easily recovered after utilization by chemical cleaning procedures or mechanical back flushing [18].

In the work by Espejo-Carpio et al. [19], ceramic membranes were employed satisfactorily as a separation stage in an integral process to produce ACE inhibitory peptides from goat milk proteins. A complete pore blocking model was assumed to explain the time evolution of the permeate flux. In the previous hydrolysis, subtilisin and trypsin were employed both in individual and simultaneous modes for 3 h.

The aim of the present paper was to study the hydrolysis and filtration stages in order to produce hydrolysates with enhanced ACE inhibitory activity. Instead of individual use of the enzymes, the two possible sequential treatments were assayed for longer reaction times of 5 h. The filtration stage was studied by employing two ceramic membranes of different MW cut-off. The improvement in the ACE inhibitory activity of permeates in comparison to the feed was analyzed. Finally, the fouling mechanism occurring during the filtration was evaluated in detail by testing the fitting capabilities of a total of four models. In addition of complete pore blocking, the following mechanisms were also considered: internal pore blocking, partial pore blocking, and cake formation.

2. Materials and methods

2.1. Enzymes and substrates

The enzymes assayed in this work were two serine endoproteases: subtilisin (EC 3.4.21.62) and trypsin (EC 3.4.21.4). Both commercialized as Alcalase 2.4L and PTN 6.0S by Novozymes (Denmark), respectively. These enzymes were combined and employed to hydrolyze the two families of proteins in milk: caseins and whey proteins. In order to obtain these protein fractions, goat milk fat was removed by centrifugation, at 4,800 g and 4˚C. Subsequently, the skimmed milk obtained was filtered through a 0.14 μm ceramic membrane (Tami, France). The caseins remained in the retentate, while whey proteins could cross the membrane being recovered in the filtrate. Therefore, the filtrate was composed of whey proteins, while retentate was mostly composed of caseins, although a small proportion of whey proteins could be present. The casein and whey protein solutions employed as

substrates presented a protein concentration of 64.3 and 2.5 g/L, respectively.

2.2. Hydrolysis of milk proteins

The hydrolysis of goat milk proteins was studied by subjecting each protein fraction to three different enzymatic treatments. In the first treatment (A), the proteins were firstly hydrolyzed by subtilisin for 2.5 h; then, trypsin was added to the reactor and the hydrolysis continued for 2.5 h more. In the second treatment (B), trypsin was added at the begging and subtilisin was incorporated to the reactor after 2.5 h. In the third treatment (C), subtilisin and trypsin were added simultaneously at the beginning of the reaction. In all the cases, hydrolysis was stopped after 5 h of reaction by thermal denaturalization of the enzyme.

Substrate solutions were hydrolyzed at 50°C and pH 8 in a stirred tank reactor. The enzyme concentration was 5 and 0.5 g/L for caseins and whey protein hydrolysis, respectively. The pH was controlled by an automatic titrator (718 Stat Titrino, Metrohm, Switzerland). The amount of sodium hydroxide (1 M) added to maintain constant pH was related to the degree of hydrolysis (proportion of cleaved peptide bonds) according to the pH-stat method [20].

$$\mathrm{DH} = B \cdot N_b \cdot \frac{1}{\alpha} \cdot \frac{1}{\mathrm{MP}} \cdot \frac{1}{h_{tot}} \cdot 100 \quad (1)$$

where B is volume of NaOH consumed, N_b is base normality, and MP is the mass of the protein. According to Adler-Nissen [20], the parameters $1/\alpha$ (the inverse of the average degree of dissociation of the α-NH group) was 1.13 and h_{tot} (total number of peptide bonds in the protein substrate) was 8.8 and 8.2 meq/g protein for caseins and whey proteins, respectively. Samples of 0.5 mL were drawn every hour to evaluate the ACE inhibitory activity. Pearson product-moment correlation coefficient was employed to compare the values of ACE inhibitory activity and degree of hydrolysis.

2.3. Filtration of hydrolysate

Two different modules were assayed in order to evaluate the performance of ultrafiltration ceramic membranes in the improvement of the ACE inhibitory activity of the hydrolysates. The experiments were carried out in the batch concentration mode using Inside Ceram tubular modules (Tami, France) with MW cut-offs of 50 and 15 kDa. Temperature in the feed tank was kept at 50°C by using a thermostatic bath. Filtrations were performed employing a transmembrane pressure of 1 bar and a cross-flow velocity of 3.3 m/s. The ultrafiltration was followed until reaching a concentration factor of 3.

Subsequently, the membrane was subjected to a cleaning stage with a solution of 20 g/L of sodium hydroxide with 2 g/L of sodium dodecyl sulfate as surfactant agent. The operational conditions of the cleaning were: temperature at 50°C, transmembrane pressure 1 bar, and cross-flow velocity 3.3 m/s. The cleaning efficiency was calculated as:

$$E = \frac{(R_0 - R_{\mathrm{NaOH}})}{(R_0 - R_{\mathrm{M}})} \cdot 100 \quad (2)$$

where R_M is the intrinsic resistance of the virgin membrane, R_0 is hydraulic resistance after the filtration, and R_{NaOH} is the hydraulic resistance after the cleaning. Each resistance was calculated as the inverse of the slope when water flux is calibrated against transmembrane pressure.

2.4. Cross-flow filtration models

Fouling could be classified according to the blocking mechanism into complete blocking, internal pore blocking, partial blocking, and cake filtration. These four mechanisms can be expressed mathematically by one unified equation [21]:

$$-\frac{\mathrm{d}J}{\mathrm{d}t} = k_n (J - J^*) J^{2-n} \quad (3)$$

where J^* is the critical flux, which could be considered as the flux attained at steady state (J_{lim}) [22,23]; k_n is a phenomenological parameter, and n is a general index which depends on fouling mechanism. Indeed, depending on the value of n, Eq. (3) becomes into four different equations, which are representative of four possible fouling mechanisms [21].

2.4.1. Complete pore blocking ($n = 2$)

It is observed when the molecules are larger than the pores. In this case, the membrane pores would be sealed by these molecules as a consequence of a complete pore obstruction. The resulting equation is:

$$J = J_{lim} + (J_0 - J_{lim}) \cdot e^{-k_2 \cdot t} \quad (4)$$

where J is the permeate flux, J_0 is the initial permeate flux ($t = 0$).

2.4.2. Internal pore blocking ($n = 1.5$)

When molecules are small enough to enter the pores, their deposition or absorption inside the pores leads to a reduction in pore volume. In this case, fouling becomes independent of cross-flow velocity and no limiting value for the flux may be attained, therefore, $J_{lim} = 0$. The resulting equation is:

$$\frac{1}{J^{0.5}} = \frac{1}{J_0^{0.5}} + k_{1.5} \cdot t \tag{5}$$

2.4.3. Partial pore blocking ($n = 1$)

It is usually observed when molecules and pores have similar sizes. As in the complete pore blocking, the molecules could block the pores, although such blocking is not permanent. The molecules can also obstruct the entrance of the pore without completely blocking it.

$$k_1 \cdot t = \frac{1}{J_{lim} \cdot \ln\left[\left(\frac{J_0 - J_{lim}}{J_0}\right) \cdot \left(\frac{J}{J - J_{lim}}\right)\right]} \tag{6}$$

2.4.4. Cake formation ($n = 0$)

A layer composed by big molecules, which cannot enter into the pores, is formed on the surface of the membrane. The equation is:

$$k_0 \cdot t = \frac{1}{J_{lim}^2} \cdot \ln\left[\left(\frac{J}{J_0} \cdot \frac{J_0 - J_{lim}}{J - J_{lim}}\right) - J_{lim}\left(\frac{1}{J} - \frac{1}{J_0}\right)\right] \tag{7}$$

2.5. Determination of ACE-inhibitory activity

A spectrophotometric assay [24] was used to determine the inhibitory activity of the hydrolysates. The method is based on the linear decrease of absorbance (340 nm) observed during the hydrolysis of the tripeptide N-[3-(2-N-[3-(2-Furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) with ACE (EC 3.4.15.1), both from Sigma-Aldrich (USA). The slope of this descent is related to the enzymatic activity of ACE. The percentage of ACE inhibition is calculated taking into account that the slope of the sample (p_i) in the absence of inhibitor (p_0) corresponds with 100% enzyme activity:

$$\mathrm{ACE}_{\mathrm{inhibition}}(\%) = \left(\frac{p_i}{p_0}\right) \cdot 100 \tag{8}$$

The reaction was carried out in a 96-well microplate at 37°C for 30 min in a Multiskan FC microplate photometer (Thermo Scientific, Finland). Casein hydrolysates samples were diluted 1:50 before analysis, while whey protein hydrolysate samples were analyzed directly. Samples were analyzed in triplicate. An inhibition index (I_{ACE}) was calculated to compare samples with different protein concentration by dividing the percentage of ACE inhibitory activity over the protein concentration of the sample.

3. Results and discussion

3.1. Hydrolysis of milk proteins

Figs. 1 and 2 show the hydrolysis curves as well as the percentage of inhibition for caseins and whey protein hydrolysis, respectively.

3.1.1. Variation of degree of hydrolysis

In all the experiments, at the beginning of the reaction a constant steep increase in the degree of hydrolysis was observed. After this initial period, the reaction rate decreased. However, when fresh enzyme was incorporated (treatment A and B) a new period of constant rate was observed, which means that new bonds were being cleaved. Similar results were found in other hydrolysis studies employing sequential addition of enzymes [25,26]. The final DH reached after 5 h varied from 33 to 36% and from 22 to 28% for caseins and whey proteins, respectively. In general, caseins were hydrolyzed more extensively than whey proteins, this can be explained because of the higher resistance of globular proteins, such as whey proteins, to the action of many enzymes [27].

In the case of sequential addition of subtilisin and trypsin (treatments A and B), the results pointed that the treatment A gave rise to the higher DH values for both substrates. Similar results were reported previously for the sequential hydrolysis of fish proteins with subtilisin and trypsin [26]. This suggests that subtilisin, which is able to cleave a broader spectrum of peptide bonds, could enhance the activity of trypsin by making accessible new peptide bonds that can be hydrolyzed by trypsin. Nevertheless, the highest DH was always obtained for the simultaneous hydrolysis of subtilisin and trypsin (treatment C).

3.1.2. Variation of ACE inhibitory

The ACE inhibitory activity of the caseins and whey protein hydrolysates is presented in Figs. 1 and 2, respectively. As can be observed, there were differences between treatments in the evolution of ACE inhibitory

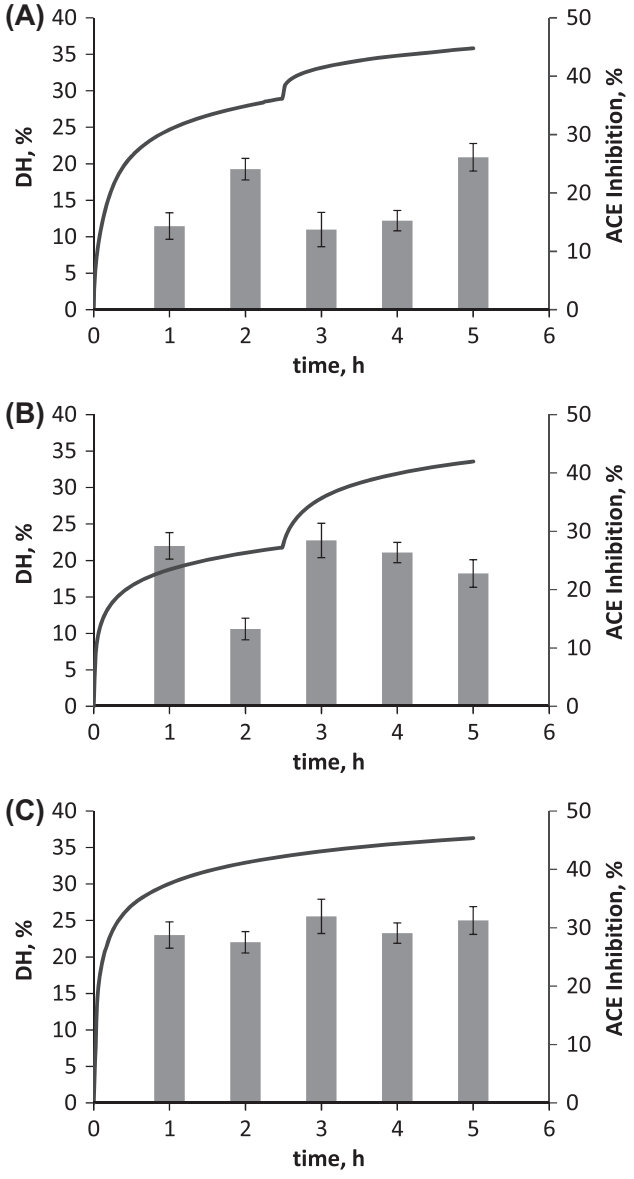

Fig. 1. Degree of hydrolysis (line) and ACE inhibitory activity (bar) of the hydrolysis of caseins for the sequential hydrolysis with subtilisin and trypsin (A); the sequential hydrolysis with trypsin and subtilisin (B); the simultaneous hydrolysis with both enzymes (C).

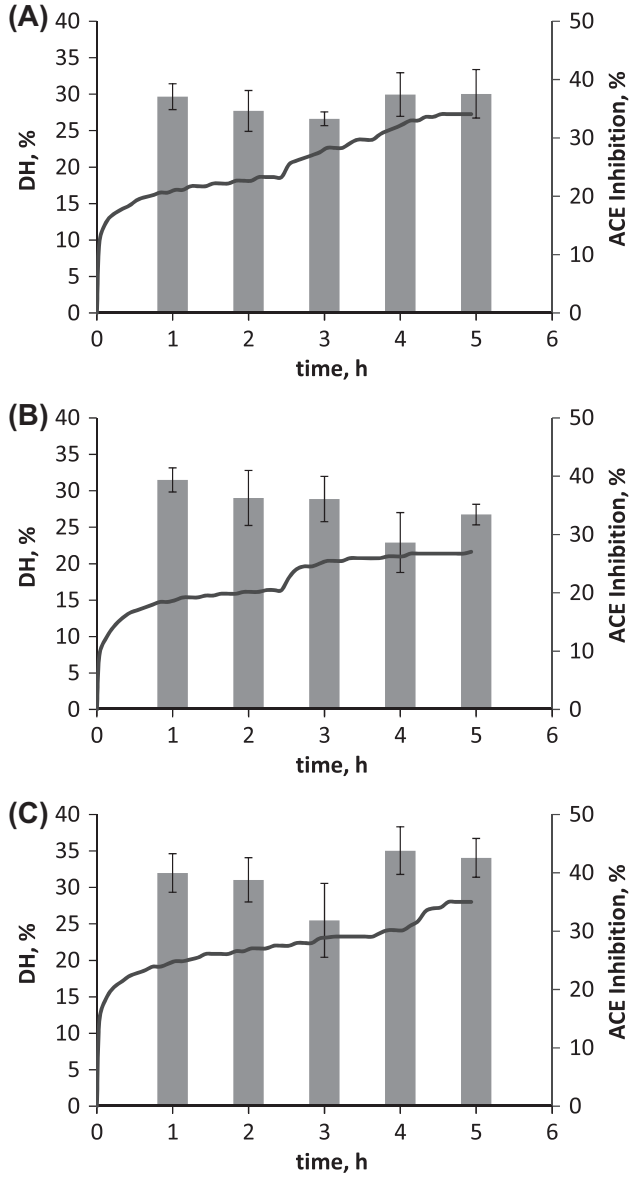

Fig. 2. Degree of hydrolysis (line) and ACE inhibitory activity (bar) of the hydrolysis of whey proteins for the sequential hydrolysis with subtilisin and trypsin (A); the sequential hydrolysis with trypsin and subtilisin (B); the simultaneous hydrolysis with both enzymes (C).

activity, especially in the case of caseins. When subtilisin was added firstly in the hydrolysis of caseins (Fig. 1(A)), the ACE inhibition increased progressively, but when trypsin was incorporated, the inhibition declined. In this case, trypsin seemed to degrade the active peptides generated previously by subtilisin. This has been described before for the hydrolysis of yak caseins [7]. Only after 5 h of reaction, trypsin was able to generate new inhibitors which increased the inhibitory activity. In the case of trypsin being added at the beginning (Fig. 1(B)), the inhibitory activity increased initially and then decreased with time. This has been also observed in the hydrolysis of whole milk [28]. When subtilisin was added, new inhibitory peptides were generated and the ACE inhibitory activity increased considerably. Finally, the ACE inhibitory activity during the simultaneous hydrolysis of caseins remained at higher values and no statistical differences were found between the samples obtained at different reactions times (p-value = 0.323). This could be

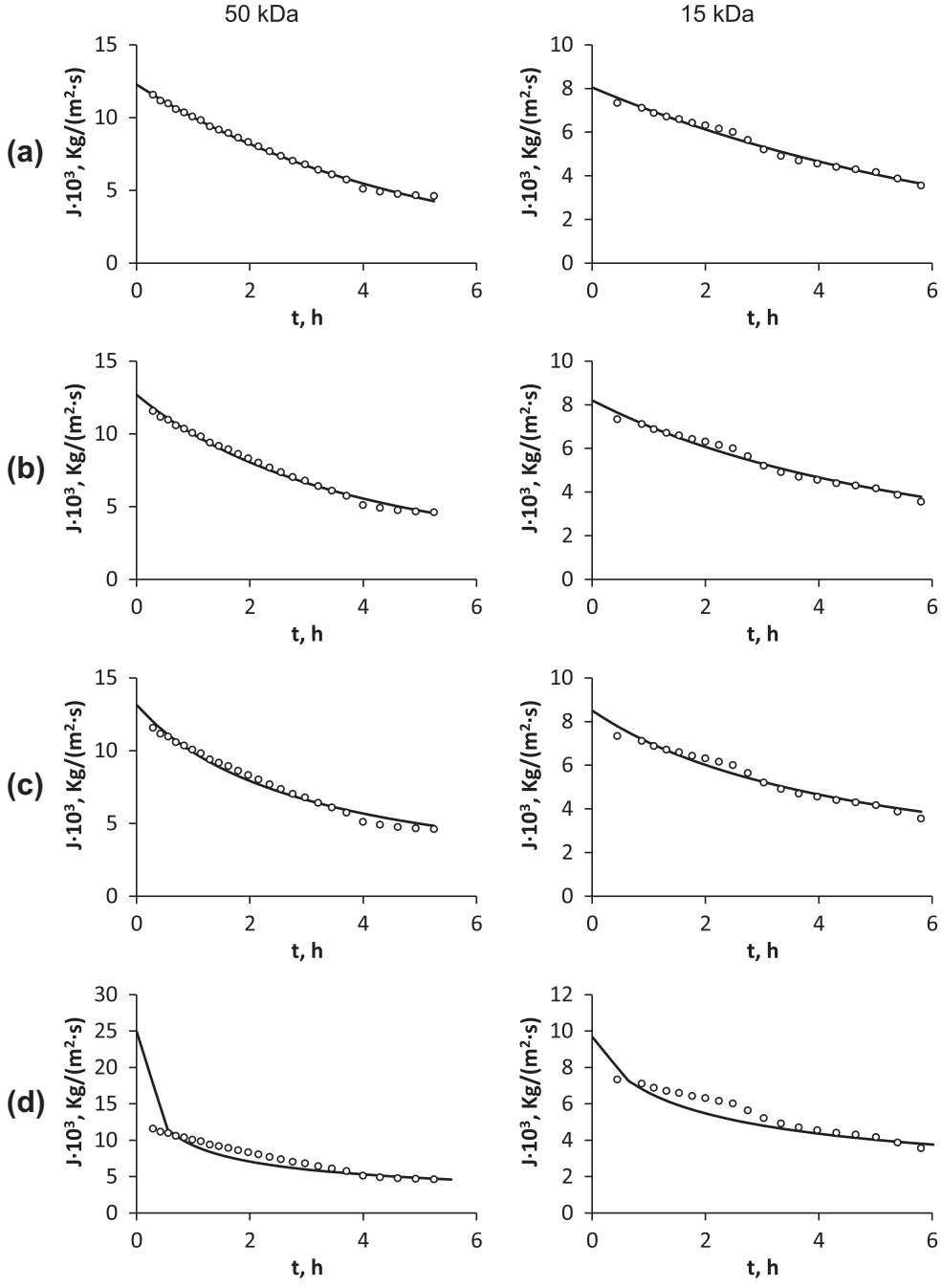

Fig. 3. Observed (circle) and predicted (line) flux of permeate for the 50 and 15 kDa membranes. The following models were considered: complete pore blocking (a), internal pore blocking (b), partial pore blocking (c), and cake formation (d).

explained since the generation of new active peptides would be offset by the degradation of some of the active peptides previously generated. Similar behavior have been found previously in studies of protein hydrolysis employing mixtures of enzymes [26].

The changes observed in ACE inhibitory activity during the hydrolysis of whey proteins were less pronounced than in the caseins hydrolysates. In all the treatment tested (Fig. 2), after an initial increase of the inhibitory activity, it remained almost constant. Indeed, no statistical differences were found for the samples of the same treatment (p-value < 0.05). Therefore, contrary to what was observed for caseins, the addition of the secondary enzyme did not imply a

variation in activity (Fig. 2(A) and (B)). These results are different from those report by Wang et al. [11] in the hydrolysis of whey protein isolate with subtilisin and bacillolysin.

For both substrates, the treatment yielding the best inhibitory capacity was the simultaneous hydrolysis with subtilisin and trypsin (treatment C). In order to compare the inhibitory activity of the two protein fractions, it is necessary to take into account the amount of protein present in each sample. As mentioned above, caseins hydrolysate samples were diluted before analysis obtaining a protein concentration of 1.29 mg/mL, while whey protein hydrolysates presented a protein concentration of 2.5 mg/mL. The average value of the inhibitory index (I_{ACE}) of the samples of treatment C was 22.9 and 15.7%/($mg_{protein}$/mL) for caseins and whey proteins, respectively. Therefore, the peptides present in caseins hydrolysate would have better potential as inhibitors, since they were able to reduce more efficiently the activity of ACE for the same protein concentration. For this reason, the hydrolysate of caseins produced by the simultaneous action of subtilisin and trypsin was selected for membrane ultrafiltration. Specifically, the hydrolysate of 3 h was preferred since the ACE inhibitory activity was the highest. Moreover, after 3 h, the degree of hydrolysis had almost reached its maximum value. Consequently, it would not worth maintaining the reaction two more hours. Since most active peptides identified present peptides chain between 2 and 12 amino acids [5], an extensive degree of hydrolysis would be desirable. Indeed, the selected 3-h hydrolysate had a DH of 34.4%, which implies an average peptide length chain of three amino acids approximately [20].

When comparing the ACE inhibitory values with DH, it was observed that values for the correlation coefficient were usually below 0.35. This lack of correlation has been also observed previously in other hydrolysis studies [7,29]. It could be explained because the ACE inhibitory activity depends on the sequence of the peptide more than on the size of the peptide.

3.2. Filtration of hydrolysate

A caseins hydrolysate obtained by simultaneous treatment with subtilisin and trypsin for 3 h was ultrafiltered through 50 and 15 kDa tubular ceramic membranes.

3.2.1. Evolution of the flux

Fig. 3 shows the evolution of permeate flux for the filtrations through 50 and 15 kDa membranes. In both cases, the permeate flux decreased with time at decreasing rate, without reaching a steady state during the operation. This deceleration in flux decline is more clearly observed in Fig. 4, where, after reaching the concentration factor of 2, the flux decreased more slowly until reaching a concentration factor of 3.

The membrane of 50 kDa had a flux decline of 60.3% during the 5.2 h of operation, the flux varied from 11.56×10^{-3} to 4.59×10^{-3} kg/(m^2 s). On the other hand, the flux for the 15 kDa membrane was modified from 7.33×10^{-3} to 3.55×10^{-3} kg/(m^2 s) after 5.8 h of operation, which implied a lower flux decline (51.5%). In both the cases, a concentration factor of 3 was reached, but the 50 kDa membrane was more affected by fouling. Higher values of initial flux (13.6×10^{-3} kg/(m^2 s)) were reported for the ultrafiltration through 8 kDa of tuna hydrolysates [30].

Experimental flux data were fitted by non-linear regression to the four fouling models proposed. Fig. 3 shows the experimental data together with the model output. The fitting parameters of each model are presented in Table 1, where it is also shown the coefficient of determination (R^2). The model of complete pore blocking had the best fit to the experimental fluxes for 50 and 15 kDa membranes. The value of J_{lim} was low in both cases, being zero for 15 kDa membrane. Therefore, according to this model, the membrane would be fouled progressively until total clogging occurs. This seems reasonable since a continuous reduction in the flux was observed experimentally. According to the parameter k_2, the theoretical rate of fouling for 50 kDa was higher than for 15 kDa. This was confirmed by the larger flux decline registered in the filtration through 50 kDa.

Internal and partial pore blocking, also gave acceptable fittings for both 50 and 15 kDa membranes. Probably, the three mechanisms of pore blocking (complete, internal, and partial) occurred during these

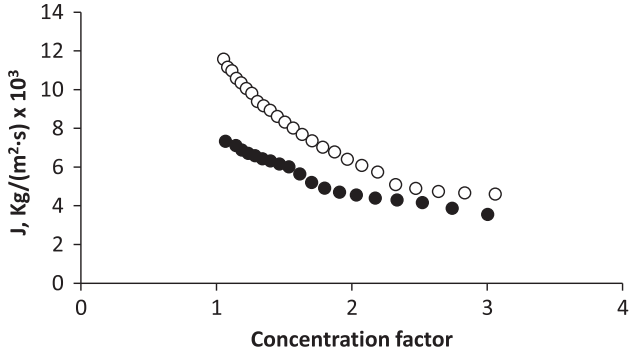

Fig. 4. Evolution of permeate flux versus concentration factor for 50 (○) and 15 kDa (●) membranes.

Table 1
Parameters of the adjusted model for the cross flow ultrafiltration of casein hydrolysate through ceramic membranes of 50 and 15 kDa

Cut-off (kDa)	Fouling model	J_0, kg/(m^2 s)	J_{lim}, kg/(m^2 s)	k_n	R^2
50	Complete	1.22E-2	3.91E-5	5.64E-5	0.9966
	Internal	1.26E-2	–	1.13	0.9926
	Partial	1.31E-2	1.50E-4	7.08E-3	0.9818
	Cake	2.49E-2	4.38E-4	2.13	0.9307
15	Complete	8.05E-3	0	3.80E-5	0.9849
	Internal	8.20E-3	–	2.24E-4	0.9784
	Partial	8.50E-3	7.57E-5	6.83E-3	0.9660
	Cake	9.68E-3	5.71E-5	2.04	0.9353

filtrations. However, the complete pore blocking seemed to be the predominant fouling mechanism, which may be due to the presence of macromolecules in the feed. Finally, the model of cake formation gave the worst fitting results for both the membranes.

3.2.2. Membrane cleaning

After the filtrations, the membranes were cleaned with sodium hydroxide solution. In order to evaluate the recovery of the filtration characteristic, the resistance of the membrane before the filtration (intrinsic resistance), the resistance after the filtration process and after cleaning process, were determined. The total resistance increased after the filtration process above 94%, in both filtrations. However the cleaning efficiency obtained was 97 and 99%, for 50 and 15 kDa membranes, respectively. Consequently, in both cases, the cleaning protocol was able to remove the fouling which implied that membranes could be employed again in another filtration cycle. These results are in line with those by Blanpain-Avet et al. [31] who assessed the efficiency of sodium hydroxide cleaning solutions in the removal of whey protein fouling over ceramic membranes.

3.2.3. Variation of ACE inhibitory activity after filtration

ACE inhibitory activity and protein concentration were evaluated in the feed solution as well as both in the final retentate and permeate. Again, the I_{ACE} was used for comparison between the samples. The filtration through 50 and 15 kDa worsened the inhibitory activity of the retentate due to the concentration of large peptides, which are usually less active. The I_{ACE} decreased 60 and 55% with respect to the feed for 50 and 15 kDa retentates, respectively. On the contrary, the small peptides which passed through these membranes improved the ACE inhibitory activity of the permeate fractions. In the case of 50 kDa, the improvement reached the 42% while 15 kDa originated an increase of 23%.

Previous studies have also produced permeates with enhanced activity after ultrafiltration of protein hydrolysates. Pihlanto-Leppälä et al. [10] obtained an average improvement of 22 and 32% in ACE inhibitory activity for 30 and 1 kDa permeates, respectively, from a bovine whey protein hydrolysate. Faria et al. [32] found that the permeate of a collagen hydrolysate processed consecutively with 50 and 1 kDa membranes exhibited better ACE inhibitory activity *in vivo* than that filtered through 8 kDa.

4. Conclusions

Casein and whey protein fractions from goat milk were successfully hydrolyzed by combinations of subtilisin and trypsin. The ACE inhibitory activity of the hydrolysates seemed to be more influenced by the substrate and enzyme treatment employed than for the degree of hydrolysis. Caseins hydrolyzed simultaneously for 3 h with subtilisin and trypsin gave the most potent ACE inhibitory hydrolysates. The ultrafiltration of this hydrolysate through 50 and 15 kDa tubular ceramic membranes allowed the obtention of permeates with an improved inhibitory activity (42 and 23%, respectively). During the filtrations, permeate flux decreased continuously because of the fouling, which was fitted to an equation model corresponding to the complete pore blocking mechanism. The cleaning procedure with a sodium hydroxide solution was able to restore the initial permeability of the membrane.

Acknowledgments

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Nomenclature

DH	—	degree of hydrolysis (%)
B	—	volume of NaOH solution consumed (mL)
N_b	—	normality (meq/mL)
α	—	average degree of dissociation of the α-NH group
h_{tot}	—	total number of peptide bonds in the protein substrate (meq/g)
MP	—	protein mass (g)
R	—	hydraulic resistance of the membrane (kg/bar m^2 s)
J	—	permeate flux (kg/m^2 s)
J^*	—	critical flux in modified complete blocking law (kg/m^2 s)
n	—	general index (units depend on mechanism)
k_n	—	phenomenological parameter (units depend on mechanism)
p	—	slope of the variation of absorbance in ACE inhibitory assay
I_{ACE}	—	inhibition index (%/(mg/mL))

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