

Research Article

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Comparison of selective hydrolysis of α -lactalbumin by acid Protease A and Protease M as alternative to pepsin: potential for β -lactoglobulin purification in whey proteins

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Abstract

The experiments reported in this research paper examine the potential of digestion using acidic enzymes Protease A and Protease M to selectively hydrolyse α -lactalbumin (α -La) whilst leaving β -lactoglobulin (β -Lg) relatively intact. Both enzymes were compared with pepsin hydrolysis since its selectivity to different whey proteins is known. Analysis of the hydrolysis environment showed that the pH and temperature play a significant role in determining the best conditions for achievement of hydrolysis, irrespective of which enzyme was used. Whey protein isolate (WPI) was hydrolysed using pepsin, Acid Protease A and Protease M by randomized hydrolysis conditions. Reversed-phase high performance liquid chromatography was used to analyse residual proteins. Regarding enzyme selectivity under various milieu conditions, all three enzymes showed similarities in the reaction progress and their potential for β -Lg isolation.

Whey proteins such as β -lactoglobulin (β -Lg) and α -lactalbumin (α -La) have found use in functional foods and beverages, infant formulas and sport diets as well as being a very good source of bioactive peptides (Chatterton *et al.*, 2006; Smithers, 2008; Cheison *et al.*, 2010; Lisak Jakopović *et al.*, 2016; Miletic *et al.*, 2016). Researchers performed numerous experiments in order to fractionate either α -La or β -Lg from whey. Specifically, Bramaud *et al.* (1997) selectively precipitated and then fractionated α -La by heating and Ca^{2+} -chelation using citrate at pH 4.2 and obtained a process which left β -Lg and other proteins in solution. Toro-Sierra *et al.* (2013) reported an improvement of that method, combined with membrane fractionation. In addition, β -Lg was separated from whey proteins at pH 2.0 in the presence of 70 g/l NaCl (Maillart and Ribadeau-Dumas, 1988; Maté and Krochta, 1994) or low pH and either NaCl or sodium hexametaphosphate (Alomirah and Alli, 2004), a precipitation process which yielded various levels of purity and protein recovery levels. These isolation methods were mainly based on the selective precipitation of either β -Lg or α -La aggregates under variation of different environmental or process parameters followed by ultra-filtration (UF). The methods, which exploit selective thermal precipitation, yield a product that is variably denatured, an undesirable side effect if applications that require native proteins are targeted. β -Lg is responsible for most of the functional properties in whey proteins (Foegeding *et al.*, 2002). Potentially, the functional properties of β -Lg are less variable if compared to whole whey proteins and it is desirable to have sufficient quantities of this important protein in as pure and native form as possible. Alternative methods are, therefore, of interest in order to develop processes that yield pure and native β -Lg fraction.

Enzymes hold great potential for the production of individual protein fractions from substrate with the minimum denaturation of desirable protein while other proteins from the substrate are fully denatured. It is already known that bovine β -Lg is susceptible to trypsin (Schmidt and Poll, 1991) and chymotrypsin (Lisak *et al.*, 2013) in a genetic-variant-dependent manner (Creamer *et al.*, 2004). Trypsin was therefore used to selectively hydrolyse β -Lg as a novel approach in order to obtain isolated native α -La (Galvão *et al.*, 2001; Custodio *et al.*, 2005; Konrad and Kleinschmidt, 2008; Cheison *et al.*, 2011). In our previous work (Lisak *et al.*, 2013) we reported that chymotrypsin shares hydrolysis similarities with trypsin with regard to the way it attacks the whey proteins. During chymotrypsin digestion of WPI, higher resistance of α -La to hydrolysis compared to β -Lg was demonstrated. Different hydrolysis behaviour for the two genetic variants of β -Lg (A and B) was observed, where β -Lg B was more resistant than β -Lg A (Lisak *et al.*, 2013). Acid Protease A is an acid proteolytic enzyme

obtained from *Aspergillus niger*. It is stable in an acid pH range of 3.0–6.0 and temperature range 30–55 °C. Its optimum is pH around 2.5 and 55 °C. Acid Protease A has a molecular weight 19 kDa. Protease M is as well acid proteolytic enzyme obtained from *Aspergillus* sp. It is stable in pH range of 3.0–6.0 and temperature range 30–55 °C. Optimal conditions are pH around 4.5 and temperature 50 °C. Acid Protease A and Protease M are derived from microbial sources and thus their price is lower, making them attractive at industrial scale.

The main goal of this research was to investigate whether usage of the microbial enzymes rather than pepsin would give the same or similar reaction properties in terms of hydrolysis of whey proteins. The hypothesis of the presented research goes in three directions: (i) is the pH influencing the susceptibility of α -La while conferring resistance to β -Lg?, (ii) is it a property of the enzymes that they are selective irrespective of the pH and (iii) a combination of both the inherent enzyme selectivity and the milieu? Thus, this work investigates whether proteases with commercial names Acid Protease A and Protease M could be applied as an alternative to pepsin hydrolysis.

Materials and methods

Materials

Whey protein isolate (WPI, 938.4 g/kg protein); obtained from Fonterra Co-operative Group Ltd. (Auckland, New Zealand) was used as the substrate for hydrolysis. The enzyme, pepsin (EC 3.4.23.1) from porcine gastric mucosa was purchased from Sigma-Aldrich (St. Louis, MO, USA). The enzymes, Acid Protease A and Protease M were purchased from Amano Enzyme Inc. (Nagoya, Japan). Bovine whey protein calibration standards: calcium depleted α -La (L6010, ≥ 850 g/kg), β -Lg genetic variant A (L7880, ≥ 900 g/kg) and β -Lg genetic variant B (L8005, ≥ 900 g/kg) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Additional reagents are mentioned under respective methods.

Methods

Calculation of the degree of hydrolysis (DH)

At acid pH values 1.5–4.0, at which the experiments were performed, amino acids are in the zwitterion state. During hydrolysis, the pH increased and addition of acid was necessary to keep it constant. HCl (0.5 mol/l) was used to keep the pH constant. The volume of HCl consumed in the reaction was used to calculate the degree of hydrolysis (DH) according to Diermayr and Dehne (1990) using Equation (1).

$$DH = \frac{F_{pH} \cdot V_{HCl} \cdot N_{HCl}}{M_p \cdot h_{tot}} \cdot 100 \quad (1)$$

where, $F_{pH} = 1/(1 - \alpha)$ is a correcting factor and is influenced by the pH value between 30 and 45 °C. The value depends on the dissociation constant of the carboxylic acid group (COOH). V_{HCl} [L] presents volume of HCl consumed during hydrolysis, N_{HCl} [mol/l] presents normality of HCl used to maintain the pH at constant experimental value; M_p [g] is mass of protein in the substrate (nitrogen content); h_{tot} is total number of available peptide bonds (in 8.8 meq/g for WPI) and α is the dissociations degree of the COOH group defined by $\alpha = 10^{pH-pK_A}/(1 + 10^{pH-pK_A})$.

Hydrolysis of whey protein isolate

WPI was dissolved in 100 mL MilliQ (MilliQ System, Millipore Corporation, Bedford, USA) purified water to 100 g/l. The pH of the solutions was adjusted using 0.5 or 1 mol/l HCl. The hydrolysis temperature was maintained by a thermostatic bath with circulation (Haake CH, Berlin, Germany) to a Schott Duran jacketed-beaker glass batch reactor (HLL Landgraf Laborsysteme, Langenhagen, Germany) which contained a magnetic stirrer. The pH of the reaction was kept constant during the hydrolysis process with the addition of 0.5 mol/l HCl using manual titration dispensed through a burette with a tap at the bottom to control the added volumes. The amount of HCl used to maintain the pH was used for the calculation of the degree of hydrolysis (DH) using the relationship in Eq. (1). Hydrolysis experiments were performed using WPI (100 g/l) at different pH values (pH 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 5.0) and different temperatures (30, 35, 40 and 45 °C). The enzyme to substrate concentration (E/S) investigated was 10 g/l. Durations of hydrolyses were ranged between 60 to 120 min. Performed hydrolyses conditions are shown in Table 1.

After pH and temperature stabilization in the reactor, a 430 μ l aliquot of the reaction mixture solution was drawn out before the addition of the enzyme, mixed with 70 μ l of 1 mol/l NaOH and marked as blank sample ($t = 0$). Then, around 93.84 mg of enzyme (pepsin, Acid Protease A or Protease M) was weighed out and dissolved in 1 mL of MilliQ water just before addition to commence the reaction. During hydrolysis, the pH was kept constant with manual titration of 0.5 mol/l HCl and the sample aliquots of 1 mL were drawn out at variable time intervals (0, 0.5, 1, 3, 5, 10, 20, 30, 60, 90 and 120 min), depending on the reaction conditions, during 60 or 120 min.

Determination of residual protein concentration (RP-HPLC)

From the aliquots drawn out at the time intervals, a definite amount was drawn out and further diluted with MilliQ water (with hydrolysis time progress sample dilution was decreased to cater for declining protein content). The samples were filtered through RC-45/25 Chromafil® Xtra Φ 0.45 μ m syringe filters (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and 1 mL placed into 1.5 mL HPLC vials (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The quantitative determination of individual protein fractions in whey was done using RP-HPLC with an Agilent 1200 (Santa Clara, CA, USA) as described by our previous work Lisak *et al.* (2013). The whole analysis process was optimized for a short analysis time of 18 min. Injection volumes were maintained at 20 μ l throughout.

Statistical analysis

All the experiments were carried out in duplicate and average values and standard deviations were calculated. The degree of hydrolysis was tested for significance by the one way analysis of variance (ANOVA) at $P < 0.05$ using SPSS 13.0 for Windows® (SPSS Inc., Chicago, IL, USA).

Results and discussion

WPI hydrolysis by pepsin

Pepsin (EC 3.4.23.1) is an acid enzyme and its optimal conditions are at pH 2.0–4.0 and it is stable to the temperature of 60 °C. Literature cites that native β -Lg is resistant to peptic hydrolysis

Table 1. Average values of residual α -lactalbumin (α -La), β -lactoglobulin B (β -Lg B), β -lactoglobulin A (β -Lg A) and the degree of hydrolysis (DH) of hydrolysed 100 g/l WPI by Acid Protease A, Protease M and pepsin at 10 g/l E/S at different temperatures (30, 35, 40 and 45 °C), pH values (1.5, 2.0, 2.5, 3.0, 3.5 and 4.0) and time of hydrolysis (60 and 120 min). (Error bars show the standard deviation, $n=2$).

Enzyme	Temperature (°C)	pH	Residual proteins (%)			Time (min)	DH (%)
			α -La	β -Lg B	β -Lg A		
Pepsin	40	2.0	36.57 ± 0.24	72.12 ± 0.87	77.23 ± 0.28	60	2.01 ± 0.03
		3.0	0 ± 0	100 ± 0	99 ± 0	60	3.67 ± 0.09
		4.0	20.18 ± 0.13	73.22 ± 0.32	77.11 ± 0.09	60	5.88 ± 0.11
	45	1.5	0 ± 0	97.00 ± 0.07	99.00 ± 0.15	60	2.13 ± 0.02
		2.5	0 ± 0	100 ± 0	100 ± 0	60	2.32 ± 0.09
		3.5	19.03 ± 0.03	97.26 ± 0.19	94.45 ± 0.31	60	5.78 ± 0.11
Acid Protease A	30	2.0	0 ± 0	80.12 ± 0.28	83.09 ± 0.15	120	1.87 ± 0.04
		4.0	0.85 ± 0.13	100 ± 0	100 ± 0	120	2.99 ± 0.09
	40	2.0	0 ± 0	99.30 ± 0.13	100 ± 0	120	2.17 ± 0.11
		4.0	3.20 ± 0.17	96.46 ± 0.22	100 ± 0	120	4.24 ± 0.07
Protease M	35	3.0	1.46 ± 0.14	94.92 ± 0.09	91.51 ± 0.22	120	2.23 ± 0.02
		5.0	12.82 ± 0.17	68.61 ± 0.13	50.31 ± 0.06	120	4.47 ± 0.09
	45	3.0	0.98 ± 0.04	100 ± 0	100 ± 0	120	2.59 ± 0.08

(Kinekawa and Kitabatake, 1996., Otte *et al.*, 1998., Konrad *et al.*, 2000). Table 1 shows the average values of the residual proteins (α -lactalbumin, β -lactoglobulin B, β -lactoglobulin A) and the degree of hydrolysis (DH) of hydrolysed 100 g/l WPI by pepsin at 10 g/l E/S, different temperatures (40 and 45 °C), pH values (1.5, 2.0, 2.5, 3.0, 3.5 and 4.0) and time of hydrolysis (60 min).

The degree of hydrolysis was calculated according to Diermayr and Dehne (1990) (Eq. 1). By increasing the pH value from 2.0 to 4.0 at the 40 °C the DH was also increased from 2.01 ± 0.03 to 5.88 ± 0.11%. The same trend occurred when reaction temperature was 45 °C. By increasing the pH value from 1.5 to 4.0 the DH was also increased from 2.13 ± 0.02 to 6.37 ± 0.12%. When the temperature was raised from 40 to 45 °C at the same pH the DH was as well increased. The highest DH was noticed at 45 °C and pH 4.0 and amounted 6.37 ± 0.12%, and the lowest DH was at the 40 °C and pH 2.0 and was 2.01 ± 0.03%. A higher DH values indicates that the hydrolysis condition was favourable for the enzymatic process. Proteins and enzymes are sensitive to pH and temperature due to their activation and inactivation effects as well as their solubility profiles. The enzyme hydrolysis was influenced by temperature and pH.

When amount of the residual proteins was assessed at pH 2.5 and 40 °C or at the pH 3.0 and 40 °C all of the α -La was hydrolysed while both genetic variants of β -Lg A and B remained unhydrolysed. When the pH value is low ($\text{pH} \leq 3.0$) tertiary structure of α -La is completely disrupted, and the secondary is partially retained and α -La binds up to 270 molecules of water and takes up the so called 'molten globule state' (Permyakov and Berliner, 2000), At these conditions β -Lg is a monomer and as well suitable substrate for hydrolysis, but pepsin selectivity was enhanced for α -La, which offers great potential for the native and pure β -Lg isolation. At pH 4.0 and 40 °C or pH 4.0 and 45 °C enzyme selectivity is lost and after 60 min of hydrolysis around 19.98% of α -La, 72.61% of β -Lg B and 79.06% of β -Lg A remains unhydrolysed. Otte *et al.* (1998) also performed hydrolysis of β -Lg with

pepsin at pH 4.0 for 22 h in order to determine the extent of degradation and concluded that native β -Lg is a poor substrate for pepsin where only 10% of β -Lg was hydrolysed.

For this research, desirable hydrolysis conditions should be that enzyme hydrolyses the α -La and β -Lg remains native and unhydrolysed. It can be concluded that the best conditions for the isolation of native and pure β -Lg by pepsin were at 45 °C and pH 2.5 and after 1 min (Fig. 1a) total hydrolysis of the α -La was obtained whereas β -Lg A and B remained intact. Similar results were reported by Kinekawa and Kitabatake (1996) who purified β -Lg from WPI at 37 °C and pH 2.0 and they concluded that native β -Lg was not susceptible to the peptic digestion. Fig. 2 depicts a typical RP-HPLC chromatogram obtained for WPI before hydrolysis (Fig. 2a) and after 60 min hydrolysis (Fig. 2b) by pepsin. Fig. 2b shows an increase in peptide accumulation and disappearance of α -La while β -Lg remains largely intact. The same trend was found for Acid protease A (Fig. 2c) and Protease M (Fig. 2d).

WPI hydrolysis by acid protease A

Table 1 shows the average values of the residual proteins (α -lactalbumin, β -lactoglobulin B, β -lactoglobulin A) and the degree of hydrolysis (DH) of hydrolysed 100 g/l WPI by Acid Protease A at 10 g/l E/S concentration, different temperatures (30 and 40 °C), pH values (2.0 and 4.0) and time of hydrolysis (120 min). By increasing the pH value from 2.0 to 4.0 at 30 °C the DH was also increased from 1.87 ± 0.04 to 2.99 ± 0.09%. The same trend was observed at 40 °C. By increasing the pH value from 2.0 to 4.0 the DH was also increased from 2.17 ± 0.11% to 4.24 ± 0.07%. When temperature was raised from 30 to 40 °C at the same pH the DH was as well increased. The highest DH was noticed at 40 °C and pH 4.0 and amounted to 4.24 ± 0.07%, and the lowest DH was at the 30 °C and pH 2.0 and was 1.87 ± 0.04%. A higher DH values indicates that the hydrolysis condition

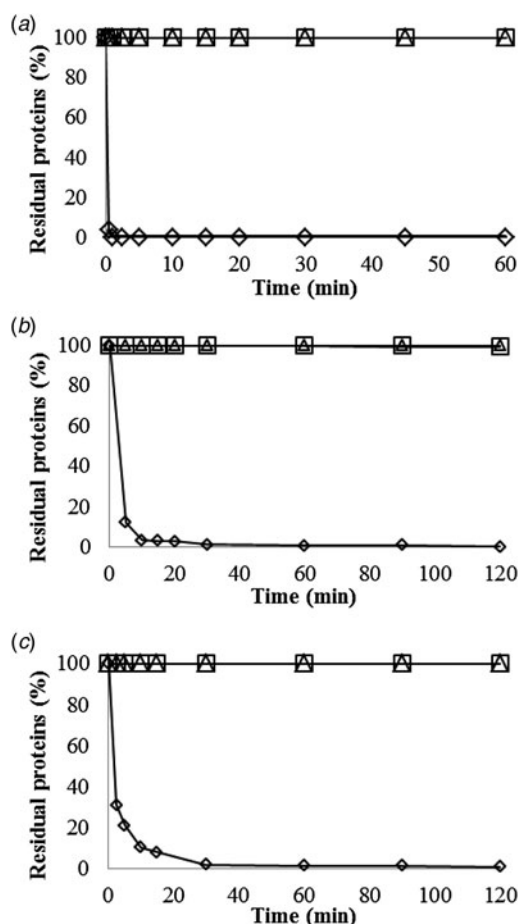


Fig. 1. Hydrolysis flow of 100 g/l WPI solution hydrolysed by: (a) pepsin (45 °C, pH 2.5, 10 g/l E/S, 60 min); (b) Acid Protease A (40 °C, pH 2.0, 10 g/l E/S, 120 min) and (c) Protease M (45 °C, pH 3.0, 10 g/l E/S, 120 min) on the residual proteins α -lactalbumin (\diamond), β -lactoglobulin B (\square) and β -lactoglobulin A (Δ) ($n=2$).

was favourable for the enzymatic process. The same DH trend was observed when hydrolysis was performed with pepsin. The obtained results are similar to findings published by Cheison *et al.* (2012). They reported that under low pH (2.5) and low temperature (30 °C), the degree of hydrolysis (DH) was lowest (1.90%), with limited hydrolysis and incomplete depletion of α -La. At higher pH of 3.5 and 45 °C, a DH of 7.35% was reached and α -La was depleted. Hydrolysis progress of WPI by Acid Protease A at 40 °C and pH 2.0 is shown in Fig. 1b. Majority of α -La (about 88%) was hydrolysed during the first 5 min of reaction. At the end of hydrolysis (120 min) all of the α -La was hydrolysed. Amounts of β -Lg A and B during the 120 min of the reaction remained unchanged, Acid Protease A did not hydrolyse β -Lg at all. By increase of the pH value, the enzymes are outside of their optimal pH milieu and the proteins, specifically α -La, are not in the hydrated molten globule state like at pH ≤ 3 and, thus, the result is the higher amount of unhydrolysed proteins.

WPI hydrolysis by Protease M

Protease M is an acidic proteolytic enzyme obtained from *Aspergillus* sp. It is stable in the pH range between 3.0–6.0 and temperatures between 30–55 °C. However, the declared optimum conditions for enzyme activity are pH 4.5 and temperature 50 °C. By increasing the pH value from 3.0 to 5.0 at 35 °C the DH was

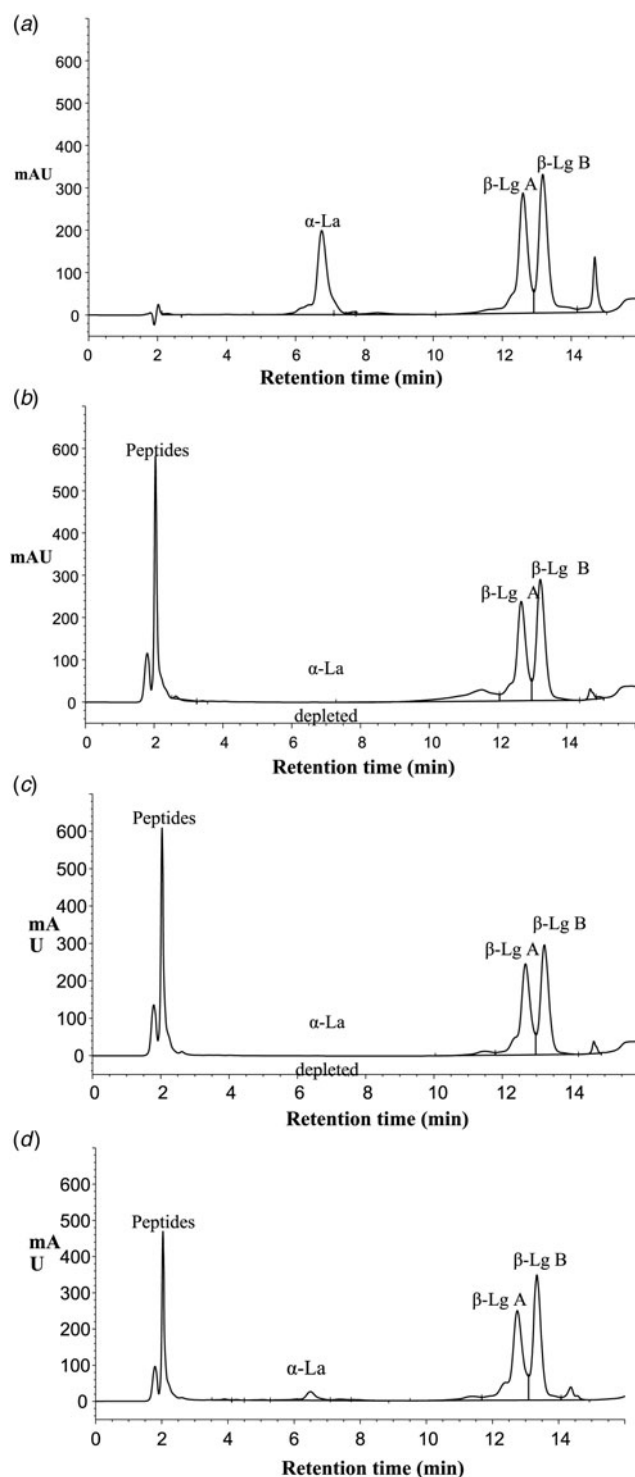


Fig. 2. Typical RP-HPLC chromatograms of whey proteins before hydrolysis at (a) time $t=0$ and after: (b) pepsin hydrolysis, $t=60$ min; (c) Acid Protease A hydrolysis, $t=120$ min and (d) Protease M hydrolysis, $t=120$ min showing α -lactalbumin (α -La), β -lactoglobulin B (β -Lg B) and β -lactoglobulin A (β -Lg A).

also increased from $2.23 \pm 0.02\%$ to $4.47 \pm 0.09\%$ (Table 1). When the temperature was raised from 35 to 45 °C at the same pH (3.0) the DH was again increased. The highest DH was obtained at 35 °C and pH 5.0 and amounted to $4.47 \pm 0.09\%$, and the lowest DH was at the 35 °C and pH 3.0 and was $2.23 \pm 0.02\%$. Increasing the temperature by 10 °C whilst keeping other environment

conditions the same achieved residual α -La of 0.48% while amounts of residual β -Lg B and β -Lg A were 5.08 and 8.49% respectively (Table 1). By increasing the pH for 2.0 pH units at the same temperature (35 °C) the amount of residual α -La increased by 11.36% while amounts of residual β -Lg B and β -Lg A decreased by 26.31% and 41.20%, respectively. The same trend was observed when hydrolysis was performed by pepsin and Acid Protease A, since all three enzymes optimally works under acidic pH.

Hydrolysis progress of WPI by Protease M at 45 °C and pH 3.0 is shown in Fig. 1c. The majority of α -La (about 92%) was hydrolysed during the first 15 min of reaction. At the end of hydrolysis (120 min) less than 1% of α -La remains unhydrolysed. Amounts of β -Lg A and B during the 120 min of the reaction remained unchanged, Protease M did not hydrolyse β -Lg at all. Thus Protease M has a great potential for isolation of β -Lg and it shows the same selectivity to proteins as pepsin and Acid Protease A. The best conditions for the isolation where β -Lg remains completely unhydrolysed and remains less than 1% α -La were at 45 °C and pH 3.0.

Comparison of WPI hydrolysis by different enzymes

Similarity between enzymes preference in protein hydrolysis order was evident. Pepsin, Acid Protease A and Protease M shows the same selectivity according to proteins when environment conditions are similar (pH 2.0–3.0 and 40–45 °C). First, pepsin, Acid Protease A and Protease M hydrolyses α -La in a short time, for 1, 20 or 30 min, respectively. Both genetic variants of β -Lg A and B remain unhydrolysed during the whole period of reaction (60 or 120 min). Influence on enzyme type on the α -La depletion over hydrolysis time is shown in Fig. 3. Fig. 3 shows that pepsin hydrolyses α -La very fast, followed by Acid Protease A and Protease M. The best environmental conditions for β -Lg isolation were in pH range 2.0–3.0 and 40–45 °C, dependent on the enzyme used. Influence of the enzyme type on the DH for the best chosen conditions for potential β -Lg isolation is shown in Fig. 4. The DH of hydrolyses were 2.32 ± 0.09 , 2.17 ± 0.11 and $2.59 \pm 0.08\%$ for pepsin, Acid Protease A and Protease M, respectively. The DH flow corresponded with the α -La depletion. The DH line for the pepsin hydrolysis had a biggest increase in first few min of hydrolysis and it achieved its maximum value 2.32% at less than 10 min. The course of reaction for Acid Protease A is very similar to the one for pepsin hydrolysis. The biggest increase in DH was in the first 10 min and the maximum value 2.17% was achieved after around 25 min of reaction. DH value for Protease M had the slowest rise until it reached its maximum value of 2.59%. The biggest increase in the DH was in the first 40 min of the reaction. The DH line of Protease M hydrolysis (Fig. 4) follows the α -La depletion (Fig. 3).

In conclusion, during the hydrolysis of 100 g/l WPI with three different acidic enzymes (pepsin, Acid Protease A and Protease M) and different milieu conditions, higher resistance of the β -Lg to hydrolysis compared to α -La was demonstrated. The enzymes of microbial origin, Acid Protease A and Protease M, showed similar selectivity as pepsin. The obtained results confirm our hypothesis and, therefore, it can be concluded that the pH value had great influence on the susceptibility of α -La, while β -Lg stayed unhydrolysed under low pH values (2.0–3.0). Furthermore, by pH change, enzyme selectivity was also changed due to the different protein susceptibilities to proteolysis at different pH values and the enzymes operating outside of their optimal conditions. Resistance to hydrolysis of β -Lg was higher at lower

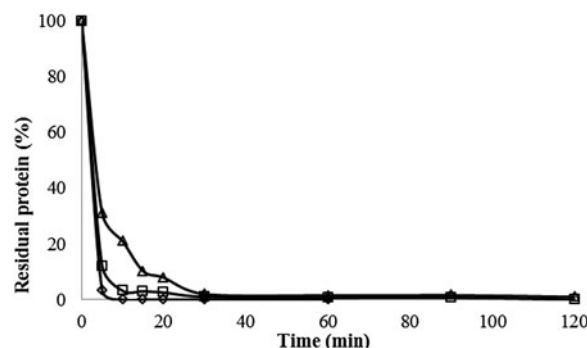


Fig. 3. Influence of the enzyme type on the α -lactalbumin depletion over hydrolysis time (60 or 120 min) at the best obtained hydrolysis conditions for pepsin (\diamond), 45 °C, pH 2.5, Acid Protease A (\square), 40 °C, pH 2.5 and Protease M (Δ), 45 °C, pH 3.0.

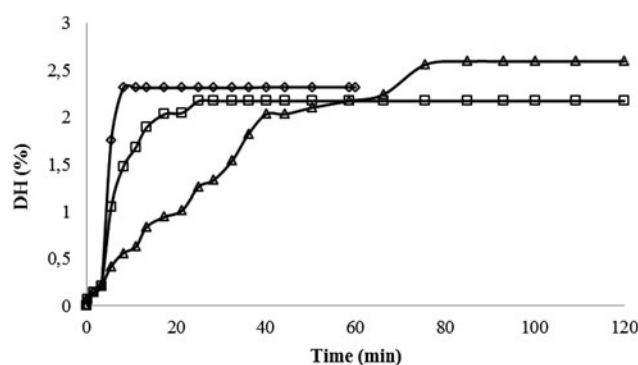


Fig. 4. Influence of the enzyme type (pepsin (\diamond), 45 °C, pH 2.5), Acid Protease A (\square), 40 °C, pH 2.5 and Protease M (Δ), 45 °C, pH 3.0) on the degree of hydrolysis (DH) at the best hydrolysis conditions for potential β -lactoglobulin isolation.

pH (2.0–3.0) and higher temperatures (40 and 45 °C) while at the same time depletion of α -La was better.

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