American Journal of Food Technology

ISSN 1557-4571

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS American Journal of Food Technology

ISSN 1557-4571
DOI: 10.3923/ajft.2016.152.158

Research Article

Whey Protein Hydrolysis with Free and Immobilized Alcalase®: Effects of Operating Parameters on the Modulation of Peptide Profiles Obtained

[1]Cecilia Fenoglio, [2]Noelia Vierling, [2]Ricardo Manzo, [2]Roberto Ceruti, [2]Guillermo Sihufe and [1,2]Enrique Mammarella

[1]Facultad de Ingeniería Química (FIQ), Universidad Nacional del Litoral (UNL), Santiago del Estero 2829, S3000AOM Santa Fe, Argentina
[2]Instituto de Desarrollo Tecnológico para la Industria Química (INTEC), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional del Litoral (UNL), Güemes 3450, S3000GLN Santa Fe, Argentina

Abstract

In this study, the effects of operating parameters on the modulation of peptide profiles obtained during enzymatic hydrolysis of Whey Protein Concentrates (WPC) with a commercial enzyme (alcalase 2.4 L AF) were analyzed. Hydrolyzates of WPC were obtained working with free and immobilized enzyme and evaluating different operating conditions (initial substrate concentrations, time, temperature and pH). Characteristics of hydrolyzates obtained were evaluated by the hydrolysis degree obtained (DH) and the peptide profile by RP-HPLC. Moreover, a quantitative descriptive sensory analysis was performed to compare different preparations of commercial desserts prepared using as ingredients milk, WPC and WPC hydrolyzates. The DH values increased very quickly during the first 10 min followed by a slow increase up to 25 min. For the range of conditions tested, we proposed to operate at temperatures close to 50°C, pH between 8 and 9 and initial substrate concentration from 7% as good reactions conditions. Finally, whey protein hydrolyzates obtained with alcalase showed very interesting sensorial characteristics to be incorporated in desserts commercial preparations.

Key words: Milk proteins, WPC, enzymatic hydrolysis

Received: March 07, 2016 Accepted: April 05, 2016 Published: June 15, 2016

Citation: Cecilia Fenoglio, Noelia Vierling, Ricardo Manzo, Roberto Ceruti, Guillermo Sihufe and Enrique Mammarella, 2016. Whey protein hydrolysis with free and immobilized alcalase®: Effects of operating parameters on the modulation of peptide profiles obtained. Am. J. Food Technol., 11: 152-158.

Corresponding Author: Enrique Mammarella, Instituto de Desarrollo Tecnológico para la Industria Química (INTEC), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional del Litoral (UNL), Güemes 3450, S3000GLN Santa Fe, Argentina
Tel: 54-342-4511595 Fax: 54-342-4511079

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Whey proteins are the soluble proteins remaining in milk after the precipitation of casein during cheese or casein production[1]. The principal whey proteins components are α-lactalbumin (α-la) and β-lactoglobulin (β-lg), which are of high nutritional quality because they are rich in essential amino acids and are an important source of bioactive sequences [1,2]. However, both of these proteins have allergic responses[3,4].

Whey protein hydrolyzates appear to be more effective because their intestinal adsorption is better, allergenicity is reduced and several active peptides are identified[4-7]. As a result of the hydrolysis, molecular properties of whey proteins change. Peptides with lower molecular weight are produced, net charge increases and more hydrophobic groups are exposed. Hydrophobic amino acids may increase the bitter taste of these products[8,9]. In this sense the hydrolysis process could produce a negative effect: The release of bitter tasting peptides. Therefore, optimization of hydrolysis process is an important aspect to obtain good quality peptides.

The most common way to produce whey protein hydrolyzates is enzymatic digestion and alcalase is one of the enzymes frequently used[10,11]. It is an inexpensive enzyme that cleaves peptide bonds with broad specificity and has been shown to hydrolyse peptides with hydrophobic amino acids such as Phe, Tyr, Trp, Leu, Ile, Val and Met at their C-terminal. Most of the studies available are limited to analyze the results of enzymatic hydrolysis of proteins under certain operating conditions and working with free soluble enzyme[6,12,13].

The immobilization of the enzyme on insoluble support lowers the costs associated to the general process and help to stabilize their structure. Selecting the most appropriate method of immobilization should be based on various performance criteria, such as maximum immobilized enzyme activity, operational stability, costs of immobilization and toxicity of immobilization reagents. Among the immobilization procedures available, the multipoint covalent immobilization seems to be one of the most promising to improve the stability of the enzyme[14]. Enzyme-support multipoint covalent attachment has proved to enhance the thermal, operational and storage stability of the immobilized enzyme compared to soluble one[15].

During enzymatic hydrolysis of whey proteins, the four major protein fractions result in multiple smaller peptide fractions, many of which again become a new kind of substrate. The evolution of this process, responds to thermodynamic variables that modulate the Michaelis-Menten constant (K_m) and maximum velocity (V_{max}), affecting the substrate specificity and thus, their selectivity. For example, while the temperature can affect the equilibrium in the formation of the enzyme-substrate complex, pH could modify the ionization state of the enzyme, substrate and enzyme-substrate complex, consequently modifying their affinity for each available substrate.

Working in a controlled manner can promote the development of certain products of hydrolysis. In general, there are scientific studies on the extent and rate of hydrolysis obtained but there are very few related to the modulation of the operating parameters and their effect on potential peptide to obtain profiles studies. For this reason, objective in the present study was to analyze and compare the alcalase 2.4 L activity to hydrolyze WPC working with the enzyme in two different conditions: Free soluble and immobilized in a insoluble support. In this way, the effect of substrate concentration, temperature of work, pH of the medium, enzyme/substrate ratio and reaction time were evaluated by determining the degree of hydrolysis (DH), the chromatographic profiles and the sensory characteristics of hudrolyzates obtained.

MATERIALS AND METHODS

Enzyme and substrate: Alcalase® AF 2.4 L (EC 3.4.21.62) from *Bacillus licheniformis* (Novozymes, Bagsvaerd, Denmark) with an activity of 2.4 U g^{-1} protein was used. A substrate for a commercial Whey Protein Concentrate (WPC) with 35% (w/w) of protein (WPC 35, Milkaut S.A., Franck, Argentina) was employed as substrate, for hydrolysis reaction.

Experimental design: The experimental design was optimized using a central composite rotatable orthogonal design with four factors: Temperature, pH, initial substrate concentration and reaction time. The experimental runs were randomized to minimize the effects of unexpected variability in the observed responses. Enzyme amount-substrate concentration ratio (E/S) was adjusted by changing the initial substrate concentration. In this way, a constant and small amount of enzyme was used in all experiments. All the experiments were carried out three times (Table 1).

Table 1: Process parameters used to obtain the hydrolyzates of whey protein

Parameters	E_1	E_2	E_3
Temperature (°C)	40	50.0	60
pH	8	9.0	10
S (% w/v)	1	7.0	13
Time (min)	10	31.6	100

S: Substrate, E_1:First experience, E_2: Second experience and E_3: Third experience

The WPC solutions at different pH were prepared dissolving the dry WPC powder in a buffer solution at room temperature by magnetic stirring during 30 min. Three WPC concentrations (1, 7 and 13% w/v) were evaluated, for solutions prepared at pH 8, 9 and 10 (using sodium phosphate buffer 40 mM and sodium carbonate buffer 40 mM). Then, pH was adjusted with 0.2 N NaOH. All other chemicals used for this task and for the control of the degree of hydrolysis achieved were of analytical grade (Cicarelli, San Lorenzo, Argentina).

Immobilization support: Chitosan with a degree of deacetylation of 85.2% was purchased from polymar (Fortaleza, Brazil). For the activation of chitosan beads was done with a 25% aqueous solution glutaraldehyde (Merck, Hohenbrunn, Germany).

Preparation of chitosan beads: The 4% (w/v) chitosan solution was prepared by dissolving the chitosan power with 5% (v/v) acetic acid solution for 24 h at room temperature. The chitosan solution was then injected into a 0.1 M NaOH solution (1:10) to form hydrogel beads under slow stirring (50 rpm) for 24 h. The beads were filtered and rinsed thoroughly, with distilled and Milli-Q water and then stored in refrigerator for further use. In this way, chitosan spheres with an average diameter of 2.1 mm were obtained.

Activation of chitosan beads: Chitosan beads were activated by suspending a mass of 10 g of carrier particles in 50 mL of a glutaraldehyde solution (5% v/v) 100 mM (pH 10) phosphate buffer. The suspension was kept under stirring at 150 rpm for 4 h at room temperature. The activated particles were filtered and rinsed vigorously with distilled water to remove excess activating agent.

Immobilization procedure: Alcalase immobilization was performed by adding 2 mL of commercial enzyme preparation to log of glutaraldehyde-activated beads suspended in 50 mL of 200 mM phosphate buffer (pH 9.0) and pouring suspension with gentle stirring (50 rpm) for 4 h at room temperature.

Hydrolysis: Enzymatic hydrolysis was performed in a 100 mL stirred batch reactor equipped with pH and temperature control. Alcalase was used in several experiments and the influence of pH was analyzed as reported in section 2.3. Hydrolysis pH was maintained at the desired value by continuous addition of 0.2 N NaOH. The degree of hydrolysis was measured by the pH-stat method, based on the consumption of sodium hydroxide needed to maintain constant the pH in the reactor of hydrolysis[16]. However, this consumption is not related in a simple way with the degree of hydrolysis achieved, being necessary to know the average pK value of the α-amino groups released during hydrolysis[17]. The hydrolysis processes with soluble enzyme were stopped by immersion of samples in a water bath at 90°C for 10 min and then immediately cooled. Finally, the hydrolyzates were stored at -20°C until RP-HPLC analysis. This procedure was carried out in different working conditions using either the enzyme free and immobilized in support particles.

Determination of the degree of hydrolysis: The degree of hydrolysis (DH), defined as the percentage of the total number of peptide bonds in a protein which have been cleaved during hydrolysis was calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis[16] as follows:

$$DH\% = 100BN_b\left(\frac{1}{\alpha}\right)\left(\frac{1}{MP}\right)\left(\frac{1}{h_{tot}}\right) \quad (1)$$

where, B is the volume of NaOH consumed (mL) to keep the pH constant during the reaction, N_b is the normality of NaOH, MP is the mass of protein (g), h_{tot} is the total number of peptide bonds in the protein substrate (meq g^{-1} protein) and α is the average degree of dissociation of the α-NH_2 groups released during hydrolysis expressed as follows:

$$\alpha = \frac{10^{(pH-pK)}}{\left(1+10^{(pH-pK)}\right)} \quad (2)$$

where, pK is the average dissociation value for the α-amino groups liberated during hydrolysis which can be defined as pK = 3.8+0.45 pH[17]. The total No. of peptide bonds (h_{tot}) in whey protein was assumed to be 8.8 meq g^{-1} [16].

RP-HPLC analysis: To analyse the peptides profiles in the samples obtained the methodology proposed by Penas *et al.*[18] with minor modifications was used. A chromatograph with a gradient programmer model 2360, a V4® variable wavelength absorbance detector (Isco, Inc., Lincoln, NE, USA) and a SynChropak RPP (250×4.6 mm) C18, 300 Å column (SynChrom, Inc., Lafayette, IN, U.S.A.) at 30°C were used. Separations were carried out at a flow rate of 1 mL min^{-1} using solvent A (0.1% trifluoracetic acid in water) for 5 min, linear gradient from 0-50% of solvent B (0.1% trifluoracetic acid in

acetonitrile) over 45 min, isocratic step at 50% B for 5 min, linear gradient to 70% B for 5 min and isocratic step at 70% B for the finals 5 min. Detection was done at λ = 214 nm. Data were processed with the Chem Research Data System Program version 3.0.2. 1994 (Isco, Inc.; Lincoln, NE; USA). One chromatogram was obtained from each sample analyzed.

Sensory analysis: A panel composed by seven trained assessors evaluated the effect of the incorporation of hydrolyzates obtained using immobilized enzyme in commercial desserts preparations. Three different preparations of commercial desserts were made using in each case as ingredients: Milk (control preparations), WPC and WPC hydrolyzates. Quantitative descriptive sensory analysis was performed to compare the different preparations, using an unstructured scale of 10 cm anchored at the ends (1 = very little; 9 = a lot) to evaluate descriptors of texture (visual consistency, creaminess, roughness and astringency) and descriptors of flavor (cream, serum, milk powder, cooked, sour, salty and sweet). Sensory assessment was conducted in individual booths at a sensory laboratory, which complies with international standards for the design of test rooms. Scores for each sample were averaged over all assessors and replicates.

RESULTS AND DISCUSSION

Influence of pH: At the beginning of hydrolysis with soluble alcalase, as pH increases, the degree of hydrolysis increases (Fig. 1) until 10 min, reaching a value of time of about 30 min after which this effect is reduced. These results obtained with alcalase 2.4 L are similar to those reported by Gonzalez-Tello *et al.*[19], working with alcalase 0.6 L; MKC Protease 660 L and PEM 2500S. The authors explained the decrease observed in the degree of hydrolysis by three factors, (a) A decrease in the concentration of peptide bonds susceptible to hydrolysis by the protease, (b) A possible inhibition of the enzymes caused by the products of hydrolysis and (c) The denaturation of the enzyme. For the range of pH analyzed, the values of DH obtained were most important for free than immobilized enzyme, the minor differences observed being for curves corresponding to pH 10 (Fig. 1).

The relationship between the presence of hydrophobic peptides and the development of bitter taste in dairy products has been reported by different authors[20,21]. In this way, Ney[20] linked the bitter taste in dairy products with the presence of peptides with high average hydrophobicity (Q above 1400), where Q is calculated taking into account the characteristics of the amino acids present in the peptide. In this study, we used a methodology based on the retention time of the more hydrophobic peptides, due to the retention of peptides in the C18 column used for RP-HPLC is a surface phenomena where hydrophobic groups from matrix column interact with those of the peptides or proteins present in the samples. So, decreasing the polarity of the mobile phase during RP-HPLC analysis, the elution of the more hydrophobic peptides is possible. In this study, a lower proportion of hydrophobic peptides (those that eluted after 40 min of chromatographic run) was observed in samples with higher values of pH (Fig. 2).

Influence of temperature: Figure 3 shows the results obtained for the different temperatures assayed. As expected, DH increases with temperature, reaching a value of time of

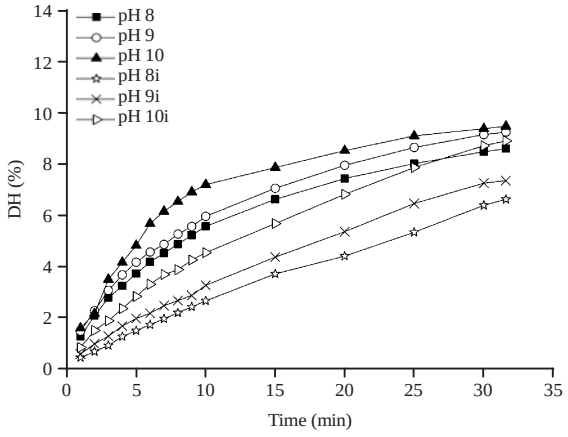

Fig. 1: The pH influence on the degree of hydrolysis at 50°C of whey proteins by alcalase 2.4 L. Initial conditions: E_0 = 40 μL, S_0 = 70 g L^{-1}

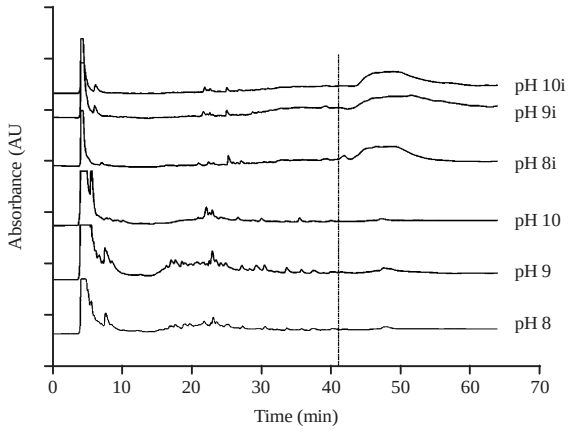

Fig. 2: The pH influence on the RP-HPLC profiles at 50°C of whey protein hydrolyzates by alcalase 2.4 L. Initial conditions: E_0 = 40 μL, S_0 = 70 g L^{-1}

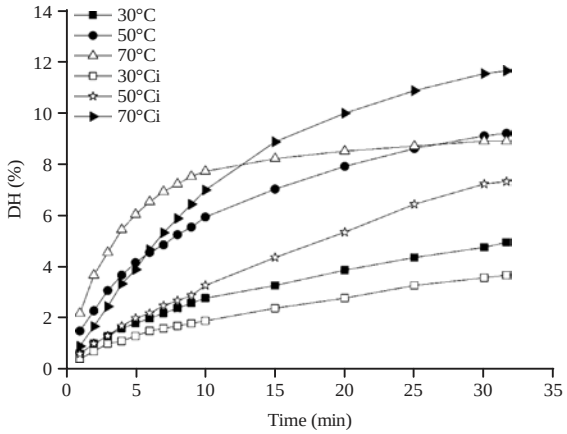

Fig. 3: Temperature influence on the degree of hydrolysis at pH = 9 of whey proteins by alcalase 2.4 L. Initial conditions: E_0 = 40 μL, S_0 = 70 g L^{-1}

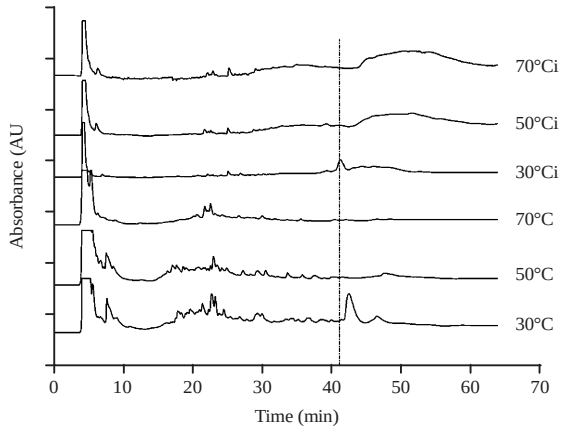

Fig. 4: Temperature influence on the RP-HPLC profiles at pH = 9 of whey protein hydrolyzates by alcalase 2.4 L. Initial conditions: E_0 = 40 μL, S_0 = 70 g L^{-1}

about 10 min after which the increasing rate is lower and a thermal inactivation effect of the soluble enzyme is observed at 70°C. On the other hand, the hydrolysis degree at 70°C with the immobilized enzyme was higher than that achieved using soluble enzyme, as a result of the thermal inactivation of the latter.

The RP-HPLC profiles obtained were influenced by temperature (major protein degradation was observed when higher temperature was used) (Fig. 4). Moreover, the most important development of hydrophobic peptides was observed at 30°C, as it can be observed in the profile, at 41.5 min, which is in agreement with results reported by other authors[2,22,23].

Influence of initial substrate concentration: As it can be observed in Fig. 5, the degree of hydrolysis decreases when

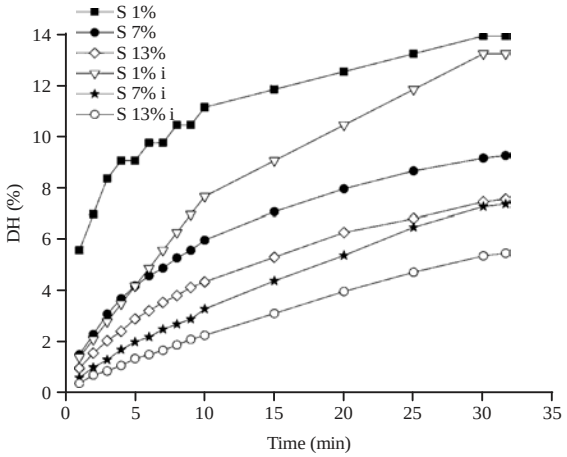

Fig. 5: Initial substrate concentration influence on the degree of hydrolysis at 50°C and pH = 9 of whey proteins by alcalase 2.4 L. Initial conditions: E_0 = 40 μL

the initial substrate concentration increases but a proportional relationship was not observed. In general terms, for the same concentration of substrate an important difference (approximately 2%) between the curves of DH for experiences with free enzyme compared to those with the immobilized enzyme was observed. As expected, the experiments with minor substrate concentration have major values of DH. Interestingly, for a substrate concentration of 7% it is possible to obtain interesting values of DH (about 6-7%) for 30 min of reaction and working both in free or immobilized form (Fig. 5).

Sensory analysis: Briefly, those desserts in which WPC hydrolyzates were incorporated showed a significant decrease in its scores of visual consistency and creaminess with respect to those that used the unhydrolyzed WPC and no differences were observed for roughness, astringency and descriptors of taste cream, milk, sour and sweet). Interestingly, for the bitter and salty descriptors was observed that while desserts made with the hydrolyzate had higher scores than those made from milk powder, were below those obtained for desserts made with WPC.

CONCLUSIONS

Reaction conditions exert a strong influence on the degree of hydrolysis and although in the chromatograms obtained for equivalent rates of hydrolysis no substantial differences in the peptide profiles developed by the enzyme degradation were observed, there were significant differences in the concentrations of the different fractions obtained. In this way, the results obtained showed that the variation of the operating conditions affected more the selectivity than the

affinity of the enzyme under consideration. Moreover, the appearance of hydrophobic peaks and hence the development of bitter taste was strongly influenced by temperature, pH and initial substrate concentration. The DH values increased very quickly during the first 10 min followed by a slow increase up to 25 min. For the range of conditions tested, we proposed to operate at temperatures close to 50°C, pH between 8 and 9 and initial substrate concentration of 7% to minimize this effect. Finally, whey protein hydrolyzates obtained with alcalase showed very interesting sensorial characteristics to be incorporated in desserts commercial preparations. In this way, new studies to evaluate nutritional characteristics for the hydrolyzates obtained could be important to complement the results obtained in this study. Finaly, the chosen immobilization technology is adequate for achieving WPC hydrolyzates with interesting properties for the formulation of food with better functional and nutritional charactesistics.

ACKNOWLEDGMENTS

This study was conducted with the financial support of Universidad Nacional del Litoral (Santa Fe, Argentina), Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina) and Agencia Nacional de Promoción Científica y Tecnológica (Argentina).

REFERENCES

1. Byrne, E.P. and J.J. Fitzpatrick, 2002. Investigation of how agitation during precipitation and subsequent processing affects the particle size distribution and separation of α-lactalbumin enriched whey protein precipitates. Biochem. Eng. J., 10: 17-25.
2. Mota, M.V.T., I.M.P.L.V.O. Ferreira, M.B.P. Oliveira, C. Rocha, J.A. Teixeira, D. Torres and M.P. Goncalves, 2004. Enzymatic hydrolysis of whey protein concentrates: Peptide HPLC profiles. J. Liquid Chromatogr. Relat. Technol., 27: 2625-2639.
3. Kananen, A., J. Savolainen, J. Makinen, U. Perttila, L. Myllykoski and A. Pihlanto-Leppala, 2000. Influence of chemical modification of whey protein conformation on hydrolysis with pepsin and trypsin. Int. Dairy J., 10: 691-697.
4. Wroblewska, B. and A. Troszynska, 2005. Enzymatic hydrolysis of cow's whey milk proteins in the aspect of their utilization for the production of hypoallergenic formulas. Polish J. Food Nutr. Sci., 14: 349-357.
5. Calvo, M.M. and R. Gomez, 2002. Peptidic profile, molecular mass distribution and immunological properties of commercial hypoallergenic infant formulas. Milchwissenschaft, 57: 187-190.
6. Guadix, A., F. Camacho and E.M. Guadix, 2006. Production of whey protein hydrolysates with reduced allergenicity in a stable membrane reactor. J. Food Eng., 72: 398-405.
7. Tolkach, A. and U. Kulozik, 2005. Fractionation of whey proteins and caseinomacropeptide by means of enzymatic crosslinking and membrane separation techniques. J. Food Eng., 67: 13-20.
8. Kim, H.O. and E.C.Y. Li-Chan, 2006. Quantitative structure-activity relationship study of bitter peptides. J. Agric. Food Chem., 54: 10102-10111.
9. Kim, H.O. and E.C.Y. Li-Chan, 2006. Application of Fourier transform Raman spectroscopy for prediction of bitterness of peptides. Applied Spectrosc., 60: 1297-1306.
10. Madsen, J.S., T.O. Ahmt, J. Otte, T. Halkier and K.B. Qvist, 1997. Hydrolysis of β-lactoglobulin by four different proteinases monitored by capillary electrophoresis and high performance liquid chromatography. Int. Dairy J., 7: 399-409.
11. Otte, J., M. Zakora, K.B. Qvist, C.E. Olsen and V. Barkholt, 1997. Hydrolysis of bovine β-Lactoglobulin by various proteases and identification of selected peptides. Int. Dairy J., 7: 835-848.
12. Guo, Y., D. Pan and M. Tanokura, 2009. Optimisation of hydrolysis conditions for the production of the Angiotensin-I Converting Enzyme (ACE) inhibitory peptides from whey protein using response surface methodology. Food Chem., 114: 328-333.
13. Ipsen, R., J. Otte, R. Sharma, A. Nielsen, L.G. Hansen and K.B. Qvist, 2001. Effect of limited hydrolysis on the interfacial rheology and foaming properties of β-lactoglobulin A. Colloids Surf. B: Biointerfaces, 21: 173-178.
14. Mendes, A.A., P.C. Oliveira and H.F. de Castro, 2012. Properties and biotechnological applications of porcine pancreatic lipase. J. Mol. Catal. B: Enzymatic, 78: 119-134.
15. Ferreira, L., M.A. Ramos, J.S. Dordick and M.H. Gil, 2003. Influence of different silica derivatives in the immobilization and stabilization of a *Bacillus licheniformis* protease (Subtilisin Carlsberg). J. Mol. Catal. B: Enzymatic, 21: 189-199.
16. Adler-Nissen, J., 1986. Enzymic Hydrolysis of Food Proteins. Elsevier Applied Science Publishers Ltd., London, Pages: 427.
17. Camacho, F., P. Gonzalez-Tello, M.P. Paez-Duenas, E.M. Guadix and A. Guadix, 2001. Correlation of base consumption with the degree of hydrolysis in enzymic protein hydrolysis. J. Dairy Res., 68: 251-265.
18. Penas, E., G. Prestamo, M.L. Baeza, M.I. Martinez-Molero and R. Gomez, 2006. Effects of combined high pressure and enzymatic treatments on the hydrolysis and immunoreactivity of dairy whey proteins. Int. Dairy J., 16: 831-839.
19. Gonzalez-Tello, P., F. Camacho, E. Jurado, M.P. Paez and E.M. Guadix, 1994. Enzymatic hydrolysis of whey proteins: I. Kinetic models. Biotechnol. Bioeng., 44: 523-528.

20. Ney, K.H., 1979. Bitterness of Peptides: Amino Acid Composition and Chain Length. In: Food Taste Chemistry, Boudreau, J.C. (Ed.). Vol. 115, American Chemical Society, USA., ISBN: 9780841205260, pp: 149-171.
21. Habibi-Najafi, M.B., B.H. Lee and B. Law, 1996. Bitterness in cheese: A review. Crit. Rev. Food Sci. Nutr., 36: 397-411.
22. Peng, X., Y.L. Xiong and B. Kong, 2009. Antioxidant activity of peptide fractions from whey protein hydrolysates as measured by electron spin resonance. Food Chem., 113: 196-201.
23. Silvestre, M.P.C., W.D.O. Afonso, C. de Oliveira Lopes Jr., V.D.M. Silva, H.A. Morais, M.W.S. de Souza and M.R. Silva, 2011. Use of subtilisin and pancretin for hydrolyzing whey protein concentrate. Am. J. Food Technol., 6: 647-660.