PERFORMANCE OF TRYPSIN MULTIPOINT IMMOBILIZED ON CHITOSAN GEL PARTICLES IN THE HYDROLYSIS OF CHEESE WHEY PROTEINS.

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Abstract

The aim of this work was to compare the performance of soluble trypsin with trypsin multipoint immobilized on chitosan gel particles in the conversion of cheese whey proteins into protein hydrolysates. It was investigated the best conditions of hydrolysis (pH and temperature) using synthetic substrate for free and immobilized trypsin: it was observed higher hydrolysis activity at 40°C and at 50°C for free and immobilized enzyme, respectively, and pH 9 for both forms of enzyme. Hydrolysis assays performed for two hours at 50°C shown that all derivatives reached from 51 to 60% of the hydrolysis degree (HD) obtained with the soluble enzyme (HD=9%). Increasing the temperature of hydrolysis by 5°C, the average hydrolysis degree obtained with derivatives activated at pH 7 and 10 were 71.84% and 70.22% of the soluble enzyme's HD, respectively. In long-term assays performed with trypsin-chitosan derivatives it was possible to reach the same HD obtained with soluble enzyme. Maximum hydrolysis were used to compare the performance of the soluble trypsin (HD=10.44% in 8 hours) with the most efficient derivative prepared – support activated at pH 7 and enzymatic load of 20mgE/gGel - (HD=10.36% in 8.5 hours).

Introduction

Nowadays, many efforts are being carried out to reduce the environmental impacts due to the discharge of highly protein industrial effluents. A way to deal with this problem consists in processing these industrial effluents in order to add value to them, that is to say transform them into raw-material for new products and processes. A very opportune example is the cheese whey, a by-product of the dairy industry, that despite its high nutritional contents (app. 0.6% of proteins and 5% of lactose, w/w), is still considered as a reject, mainly due to the large amount of generated volume and to the high costs of a proper disposal. Presently in Brazil, it is estimated that 60% of the whey is still discharged in natura directly into rivers and streams without any waste processing treatment, especially by small-scale factories.

The proteins present in this by-product have functional, nutritional, therapeutic and physiological properties. For this reason, whey proteins are used in several fields of food industry (Giraldo Zuñiga *et al.*, 2001), mainly in the formulation of dietary food supplements for children and athletes (De Wit, 1998).

Any economical process aiming at adding value to the whey must focus on two different products: lactose and protein hydrolysates. This work deals with this second product, using efficient biocatalysts in the degradation of the cheese whey proteins and with low cost.

Protein Hydrolysis

Protein hydrolysates may be obtained via acid/basic hydrolysis. These reactions, however, are unspecific, resulting in a pool of amino acids. On the other hand, enzymatic proteolysis may produce tailor-made peptides, which have higher market value. This method has been preferable to chemical methods, mainly because of the easy recovery of the catalyst after its use. For this reason, more and more the enzymatic approach becomes economically viable to be used in industrial scale.

Chitosan is a high-molecular-mass linear polysaccharide formed primarily of repeating units of β-(1-4)-2-amino-2-deoxi-D-glucopyranose (D-glucosamine) – (Sashiwa & Shigemasa, 1999). It is formed by the reaction of chitin (obtained from crab and shrimp) with concentrated alkali, being usually defined as the deacetylated derived of the chitin natural polymer (Noble *et al.*, 1999). The chitosan is an important by-product of the fishing industry and it may be used as a support to immobilize enzymes, after been submitted to some treatments (micro particulation and reticulation) for increasing its stability. It is desirable that the activation of chitosan's matrices could be performed by a simple and cheap way. In this process, the glutaraldehyde can be an adequate bifunctional agent because it makes the matrices insoluble and it also allows, controlling the reaction, high density of aldehyde groups for posterior enzyme attachment. The reticulation process drives to a structural stability of the microporos produced in the alkaline clotting of the matrices.

In this work, it was compared the performance of soluble trypsin with several derivatives of trypsin multipoint attached on chitosan gel particles in the hydrolysis of cheese whey proteins. In this context, four different trypsin derivatives were prepared, the influence of temperature and pH on the hydrolysis activity was investigated and assays of hydrolysis of whey ($10g_{protein}/L$) were developed, maintaining constant parameters such as pH of system ($pH = 8$) and enzyme/substrate ratio (E/S=1/20, w/w). The temperature (50 or 55ºC) and the reaction time (2 hours or long periods) changed depending on the experiment carried out.

Material and Methods

Material

"Minas Frescal" cheese whey was donated by Cooperativa de Laticínios São Carlos/SP. Chitosan powder was purchased from Polymar Ind. Com. Exp. Ltda (Ceará/Brazil). Trypsin (EC 3.4.21.4), extracted from bovine pancreas, was purchased from Sigma Chemical Co. Benzoyl-L-arginine-p-nitroanilide (BApNA) was purchased from Bachem AG (Budendorf, Switzerland). All other reagents were of analytical grade.

Methods

Preparation of Chitosan Gel

Chitosan gel was obtained from a solution of chitosan 2.5% (mass basis) prepared in acetic acid 5% (mass basis). This solution was filtered to remove impurities (small particles of the chitosan preparation process, from chitin) and, after that, the coagulation stage started. The filtrate was dispersed through a nozzle into the coagulation solution (1M NaOH). The system was maintained under stirring for approximately 20 minutes. The resulting suspension was filtered in a stainless steel sieve (opening of 0.177mm) and repeatedly washed with distilled water until pH 7 was reached. The wet gel was activated with glutaraldehyde using the following protocol: 1g of wet gel + 10mL of 100mM sodium bicarbonate buffer pH 10 (or 10mL of 50mM sodium phosphate buffer pH 7) + 0.7mL of glutaraldehyde (5% in mass) and stirred on a shaker for 1 hour at 25°C. Finally, the activated gel was washed with distilled water and stored at 4ºC.

Enzymatic Activity Assays

Enzymatic activity of soluble and immobilized trypsin was measured spectrophotometrically (Ultrospec 2000 - Pharmacia Biotech) following the increase of the absorbance, at λ=405nm, during the hydrolysis of the synthetic substrate N-Benzoyl-L-arginine-p-nitroanilide (BApNA). Assays were performed in a 1-cm light path glass cuvette thermostatically controlled at 25°C and adapted with magnetic stirring. 150μL of soluble trypsin solution or suspension were added to 3mL of the synthetic substrate solution (0.4mM in 50mM sodium phosphate buffer pH 8, in the presence of ethanol). The enzymatic activity was determined in Abs/min.

Immobilization of Trypsin on Chitosan Gel

Chitosan gel activated with glutaraldehyde was added to an enzymatic solution prepared in 100mM sodium bicarbonate buffer, at pH 10.05 and containing 3mM of benzamidine, in the ratio of V_{gel}/V_{total} of 1/10. This suspension was gently stirred on a shaker at room temperature (25ºC) for 24 hours. During the reaction time, aliquots of supernatant were withdrawn and its activity was measured as previously described. At the end of the period, sodium borohydride was added (1mg $NaBH_4$/mL of solution) to quench the reaction. After 30 minutes under mechanical stirring at room temperature, the produced derivative was washed with an excess of distilled water and stored at 4ºC.

Determination of the Best Hydrolysis Conditions: Temperature and pH

The best conditions of temperature and pH for the hydrolysis of the cheese whey proteins were investigated preparing solution/suspensions of trypsin in 20mM phosphate sodium buffer pH 8 and monitoring the hydrolysis of the synthetic substrate (BApNA), at λ=405nm, in specifics conditions for each assay. The best hydrolysis temperature was determined hydrolyzing the synthetic substrate (BApNA), prepared in 50mM sodium phosphate buffer pH 8, in temperatures from 30 to 70ºC as described above. The best hydrolysis pH was determined preparing solutions of BApNA in pH ranging from 6 to 10 and monitoring the hydrolysis as previously described.

Enzymatic Hydrolysis of Cheese Whey Proteins

Hydrolysis of cheese whey proteins catalyzed either by soluble or immobilized trypsin was performed in a pH-stat Titrino model (Metrohm, Switzerland), with magnetic stirring, and controlled pH and temperature. During the course of the reaction, pH was kept constant by the continuous titration of the released H^+ ions with an appropriate NaOH solution. The following equation was used to calculate the hydrolysis degree (HD) using the base consumption during the reaction (Adler-Nissen, 1986):

$$HD = V_{Base} * C_{Base} * \frac{1}{\alpha} * \frac{1}{PM} * \frac{1}{h_{tot}} * 100\% \quad (1)$$

where V_{Base} is the consumed base volume, in mL; C_{Base} is the concentration of the base, in N; α is the average degree of dissociation of R-NH groups (values supplied by Adler-Nissen for each temperature and pH); PM is the protein mass, in g, and h_{tot} is the total number of peptide bonds in the protein substrate (for cheese whey proteins: h_{tot} = 8.8 mequiv/$g_{protein}$).

Results and Discussion

Four different trypsin derivatives on chitosan gel were prepared. The immobilization process was very effective and occurred in a relatively short time: after one hour of reaction was not possible to observe any enzymatic activity in the supernatant of the suspensions and the blank (aliquot of enzymatic preparation before adding the gel) preserved 100% of its initial activity. However, long

contact periods between enzyme and solid support were used to favor the formation of additional attachments, making the immobilized enzyme molecule more rigid and, therefore, more resistant to inactivation by conformationals changes induced by heating. Table 1 shows the trypsin derivatives obtained after 24 hours of enzyme-support interaction.

Table 1: Immobilization of trypsin on chitosan gels. Experimental conditions: 25°C and pH 10.05.

pH of activation of the support	**7**		**10**	
Derivatives enzymatic load	**10mgE/gGel**	**20mgE/gGel**	**10mgE/gGel**	**20mgE/gGel**
Activity measured on supernatant in t=0 (Abs/min)	0.0442	0.0711	0.0442	0.0711
Activity measured on supernatant after 30min of reaction (Abs/min)	-	0.0114	0.0046	0.0159
Activity measured on supernatant after 24h of reaction (Abs/min)	0.000	0.000	0.000	0.000
Activity measured in the gel (Abs/min)	0.0450	0.0706	0.0453	0.0717
Recovered activity (%)	≈100%	≈100%	≈100%	≈100%

It can be observed in Table 1 that at the end of 24 hours of enzyme-support interaction, all the initial activity was detected in the suspension, resulting in 100% of immobilization yield. All derivatives presented high degree of interaction between the enzyme molecules and the support, resulting in 100% of recovery of the initial activity on the gel.

After preparation of the trypsin-chitosan derivatives, it was investigated the best conditions of temperature (from 30 to 70ºC) and pH that would supply the highest hydrolysis activity. The determination of the best hydrolysis pH was investigated using only the soluble enzyme and the trypsin-chitosan derivatives with enzymatic load of 10mgE/gGel. Figures 1(A) and (B) allow comparison between the action of soluble protease and immobilized derivatives.

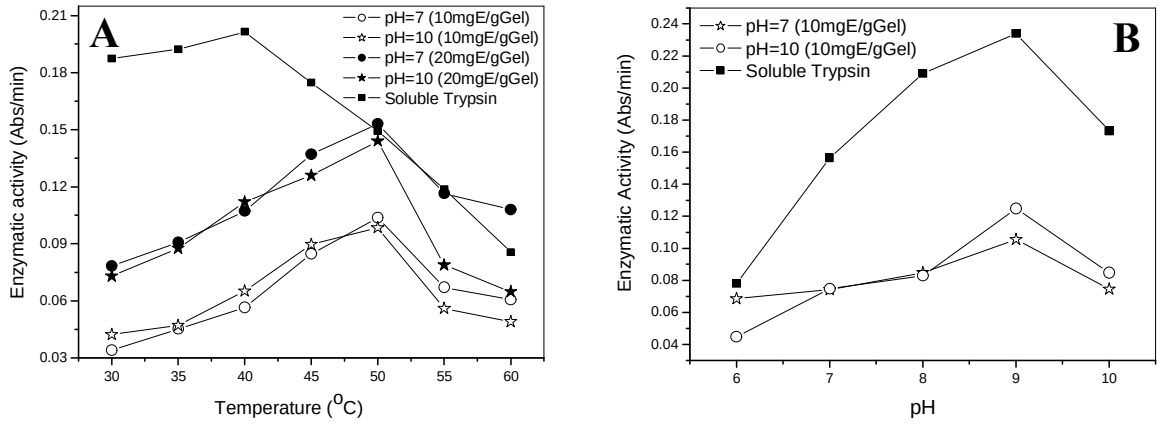

Figure 1: Determination of the best temperature (**A**) and pH (**B**) on the hydrolysis of the synthetic substrate using soluble trypsin and trypsin-chitosan derivatives.

Figure 1(A) shows that all derivatives presented the highest hydrolysis activity at 50ºC, temperature higher than the observed for the soluble enzyme (40ºC). Figure 1(B) shows that soluble enzyme and trypsin derivatives prepared on supports activated at pH 7 and 10 presented the highest hydrolysis activity at pH 9. In spite of this result, for the further experiments it was used pH 8 because this more mild condition guarantees the stability of the derivatives even for long-term assays. These data reinforce the advantages of working with immobilized enzymes; that is, it is possible to use operational conditions not advisable for free enzymes and, for this reason, in some cases the costs of operation may be reduced.

The assays of hydrolysis of cheese whey ($10g_{protein}/L$) were performed in the following experimental conditions: i) enzyme/substrate ratio (E/S) = 1/20, ii) pH = 8, iii) NaOH = 0.1M, iv) reaction time of 2 hours or long-term assays and v) temperature of 50 or 55ºC depending on the objective of the experiment.

The results of the hydrolysis assays performed at 50ºC and reaction time of 2 hours are shown in Table 2.

Table 2: Hydrolysis degrees obtained with reaction time of 2 hours and temperature of 50°C.

	Soluble enzyme	**Activation at pH 7**		**Activation at pH 10**	
Derivative enzymatic load		**10mgE/gGel**	**20mgE/gGel**	**10mgE/gGel**	**20mgE/gGel**
Time (min)	**Hydrolysis Degree (%)**				
0	0	0	0	0	0
30	7.72	2.85	3.05	2.78	2.75
60	8.33	3.67	4.20	3.63	3.66
90	8.71	4.29	4.88	4.19	4.26
120	9.02 (100%)	4.72 (52.33%)	5.41 (59.98%)	4.60 (51%)	4.69 (52%)

It can be observed in Table 2 the occurrence of delays in the action of the immobilized derivatives in relation to the soluble protease. These delays are possibly associated to diffusion limitations due to the high molecular mass of the substrates in the initial period of the reaction or to the way as polymerization of the support occurred after activation with glutaraldehyde (Arruda, 1999). It can also be observed that using derivatives more loaded (20mgE/gGel), but with same enzyme concentration in the reactor, occured an increase in the hydrolysis degree. When derivatives prepared on supports activated at pH 7 were used the resulting hydrolysis degrees were also higher. This behavior didn't happen when derivatives prepared on supports activated at pH 10 were used. In fact, it is known from literature that at pH > 9 occurs intense polymerization of glutaraldehyde on the gel surface (Monsan 1978; Sundararajan *et al.*, 1999), resulting in serious diffusion effects when these derivatives are used in the degradation of macromolecular substrates.

Aiming to reach the same hydrolysis degree obtained with free trypsin (9.02%), the system temperature was increased by 5ºC. This change led to an increase in the reaction rates. Table 3 contains the new obtained results.

Table 3: Hydrolysis degrees obtained with reaction time of 2 hours and temperature of 55°C.

	Soluble enzyme	Activation at pH 7		Activation at pH 10	
Derivatives enzymatic load		**10mgE/gGel**	**20mgE/gGel**	**10mgE/gGel**	**20mgE/gGel**
Time (min)	**Hydrolysis Degree (%)**				
0	0	0	0	0	0
30	7.90	3.39	4.24	4.24	4.13
60	8.50	4.29	5.57	5.21	5.36
90	8.75	4.98	6.48	5.76	5.94
120	8.93 (100%)	5.63 (63.05%)	7.20 (80.63%)	6.14 (68.76%)	6.40 (71.67%)

The data presented in Table 3 show that any derivative reached the hydrolysis degree of 9.02%. As before, it is possible that these experiments present a combination of diffusion and esteric effects with unfavorable orientation of enzyme active site after immobilization. Thus, there would be immobilized enzyme molecules inaccessible to the whey proteins molecules, what would delay still more the immobilized system; as a result, the real concentration of available immobilized enzyme would be smaller than the existent in the free system. However, as expected, the delays in all assays performed at 55ºC decreased.

For derivatives prepared on supports activated at pH 7, the simultaneous increases in temperature of reaction and enzymatic load led to a considerable increase in the hydrolysis degree, but it was not enough to reach hydrolysis degree of 9.02%. Aiming to overcome the system limitations (diffusion effects and high initial molecular mass of the real substrate), hydrolysis assays were performed at 55ºC and long reaction time enough to reach 9.02% of hydrolysis degree with all derivatives prepared. The results are summarized in Table 4 and they are also illustrated in Figure 2, for better visualization.

Table 4: Hydrolysis degrees obtained at 55°C and variable reaction time.

	Activation at pH 7		Activation at pH 10	
Enzymatic load / **Time (min)**	**10mgE/gGel**	**20mgE/gGel**	**10mgE/gGel**	**20mgE/gGel**
0	0	0	0	0
60	5.05	5.57	3.76	4.64
120	6.525	7.19	5.15	6.38
180	7.615	8.36	6.09	7.74
220	8.23	9.00	6.58	8.62
240	8.49	--	6.82	9.01
285	9.01	--	7.37	--
300	--	--	7.62	--
360	--	--	8.07	--
420	--	--	8.82	--
430	--	--	9.00	--

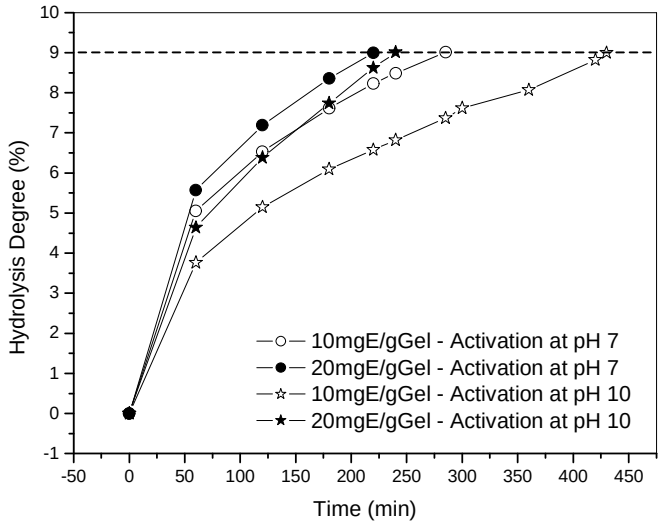

Figure 2: Hydrolysis degrees obtained at 55°C and long reaction times using different trypsin-chitosan derivatives.

Under such conditions (55ºC and long reaction periods) all four trypsin-chitosan derivatives reached the desired hydrolysis degree (9.02%). As can be seen in Figure 2, the derivatives with enzymatic load of 20mgE/gGel presented higher reaction rates. However, it should be pointed out that the derivative prepared on support activated at pH 7 with enzymatic load of 20mgE/gGel reached hydrolysis degree of 9.02% in the smallest reaction time (220 minutes) and for this reason it was chosen for the next assay.

At this point, it was investigated if the maximum hydrolysis degree that could be reached for the immobilized system would be comparable to the theoretical hydrolysis degree obtained with the soluble enzyme (Galvão *et al.*, 2001). Figure 3 shows graphically the results obtained.

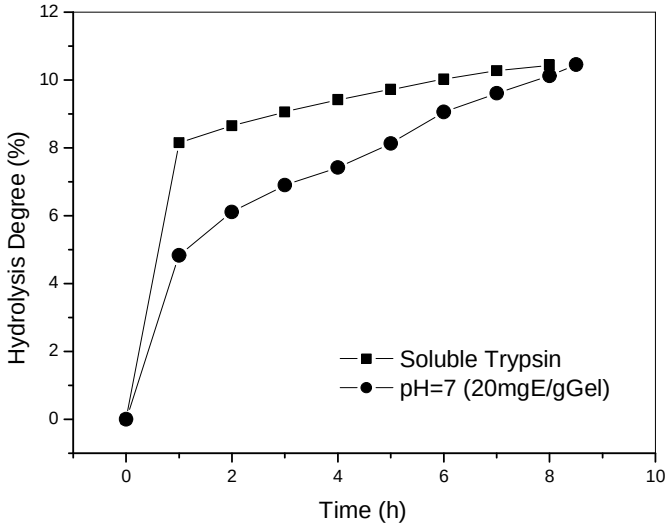

Figure 3: Maximum hydrolysis degree of cheese whey at 55°C catalyzed by soluble trypsin and derivative prepared on support activated at pH 7 (20mgE/gGel).

According to Figure 3, it was possible to confirm that diffusion effects and high initial molecular mass of the real substrate makes the immobilized system slower than the soluble one. However, with the progress of the reaction the delay was overcome by the stability of the immobilized enzyme at 55ºC and also due to the decrease of the molecular mass of the substrate. At the end of 8.5 hours of reaction, the hydrolysis degree reached wass equivalent to that obtained with the free protease. In agreement with these results, it is expected that in some operational conditions the hydrolysis degree obtainable using immobilized enzyme can be comparable to or even higher than those reached with the soluble enzyme, for example, for systems at higher temperatures.

Conclusions

After 24 hours of enzyme-support interaction all four trypsin-chitosan derivatives reached 100% of recovery of the initial activity on the gel and 100% of immobilization yield. The conditions of temperature and pH that resulted in higher hydrolysis activity were 40^{o}C and pH 9 for the soluble trypsin and 50^{o}C and pH 9 for the trypsin derivatives. The pH-stat method was adequate to follow the course of the hydrolysis reaction catalyzed by trypsin-chitosan derivatives and the determination of the hydrolysis degree was simple since its relationship with the consumed volume of base was linear. Hydrolysis assays performed for two hours at 50 and 55^{o}C shown that a combination of diffusion and esteric effects reached out to hydrolysis degree lower than those obtained with the soluble enzyme. At 55ºC and long reaction times, all four trypsin-chitosan derivatives reached the hydrolysis degree obtained with the free enzyme (9.02%). Long-term assays performed with the soluble enzyme and with the most efficient derivative prepared (derivative activated at pH 7 with enzymatic load of 20mgE/gGel) presented equivalent hydrolysis degree after approximately 8.5 hours of reaction. These results indicate chitosan as a promising alternative support for immobilization of enzymes because its versatility, cost and availability in Brazil.

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