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Immobilization of Pancreatin on Alumina and Activated Carbon for Hydrolyzing Whey

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Abstract: The immobilization of pancreatin on Alumina (AL) and on Activated Carbon (AC) at different temperatures and contact time was investigated. The amount of immobilized enzyme was estimated by subtracting the content of protein determined in the supernatant after immobilization from the amount of protein used for immobilization. Therefore, the AC had an immobilization rate of 100% for all parameters tested and the AL was much less efficient showing a maximal immobilization rate of 37% using 90 min at 25°C. The operational stability of pancreatin immobilized on the two supports was evaluated by measuring the residual activity of the enzyme after it has been used for up to 20 times to hydrolyze whey. It was observed that the pancreatin immobilized on activated carbon kept its enzymatic activity unchanged (100%) up to 5 times while on alumina it was up to 2 times.

Key words: Pancreatin, immobilization rate, operational stability, enzymatic activity, evaluation

INTRODUCTION

Enzymes have a wide variety of biotechnological, pharmaceutical, food and biomedical applications (Altun and Cetinus, 2007). However, its use as catalysts for large-scale industrial processes is limited by their high cost of production and stabilization on storage. During use, their stability decreases due to changes in pH and temperature, conformational changes as a result of friction, denaturation or inactivation and a cumulative effect of all these factors as a function of duration of their use. Moreover, since they are soluble, their recovery from a mixture of substrate and product for reuse is not economically practical rendering the process even more costly. However, the advent of immobilized enzyme technology has led to increasing efforts to replace conventional enzymatic process for immobilization (Kotwal and Shankar, 2009).

There are many advantages to attaching enzymes to a solid support. The major reasons are: multiple or repetitive use of a single batch of enzymes, the ability to stop the reaction rapidly by removing the enzyme from the reaction solution or vice versa, immobilization did have a significant effect on stabilizing the enzyme activity against the denaturing effects and the product is not contaminated with the enzyme, which is especially useful in the food and pharmaceutical industries (Ghiaci *et al*., 2009). Immobilization imparts stability to proteins by restricting the movement of the protein molecule by attachment to an inert material via chemical bonds. Therefore, the immobilized enzyme maintains its activity for a prolonged period of time when compared with free enzymes in solution (Kilinç *et al*., 2002).

The most used methods to immobilize enzymes are covalent and ionic attachment or physical adsorption (Pedroche *et al*., 2007). Adsorption is a very simple way, when compared with other immobilization methods. Basically, it involves an electrostatic and/or ionic interaction between a charged protein and the support (Tomotani and Vitolo, 2006). Moreover, by this method the immobilized enzyme has wide applicability and may provide relatively small perturbation of the enzyme native structure and function, which contributes to the maintenance of the enzyme activity (Durán *et al*., 2002).

In several studies, Tzanov *et al*. (2002), Altun and Cetinus (2007) and Ghiaci *et al*. (2009), the amount of enzyme immobilized on the support is determined by subtracting the amount of protein recovered from the supernatant and washing after immobilization with added protein, using for this, the Bradford (1976) method.

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The proteases have high cost, especially those with a high degree of purity are not reusable and have high sensitivity to various agents denaturants. Many of these undesirable characteristics can be eliminated by use of immobilization (Durán *et al*., 2002). The proteases can be immobilized without significant loss of catalytic activity and can retain this activity for a longer time that the free enzymes. Among the proteases, one can cite the pancreatin, which is an enzymatic complex containing mainly the proteases: trypsin, chymotrypsin and carboxypeptidase A (Beyon and Bond, 2001).

In this study, the pancreatin was the protease chosen to be immobilized with the purpose of its reusability in the hydrolysis of whey, since the major interest of our group is to produce dietary supplements with various clinical applications, containing hydrolyzed proteins with high nutritional value.

For estimating the operational stability of pancreatin, the Second Derivative Spectrophotometry (SDS) was chosen, because the group has been testing this technique for several purposes since many years ago. Thus, we used successfully SDS for determining the hydrolysis degree of protein hydrolysates (Silvestre *et al*., 1993), for evaluating the encapsulation rate of protein hydrolysates (Morais *et al*., 2005), as well as for estimating the Phe removal from skim milk hydrolysates (Lopes *et al*., 2005; Soares *et al*., 2006), whey hydrolysates (Delvivo *et al*., 2006), corn proteins hydrolysates (Capobiango *et al*., 2007) and rice proteins hydrolysates (Silvestre *et al*., 2009).

The aim of the present research was to study the immobilization of a pancreatin on alumina and on activated carbon, evaluating the immobilization rate and the operational stability in the hydrolysis of whey.

MATERIALS AND METHODS

L-phenylalanine, L-tyrosine, L-tryptophan, pancreatin (P-1500), alumina (A-5 type) and activated carbon (20-60 mesh) were purchased from Sigma (St. Louis, MO, USA). Whey (powder) was kindly furnished by a food producer (Minas Gerais, Brazil).

Immobilization of pancreatin: Pancreatin was immobilized by adsorption on two supports: Activated Carbon (AC) and Alumina (AL). A volume of 50 mL of the 0.1% (w v^{-1}) enzyme solution in phosphate buffer 0.1 mol L^{-1}, pH 7.5 and 10 g of the support were placed in a beaker. After 30, 60 and 90 min of contact at 25°C as well as 12 h at 5°C, the mixture was centrifuged at 11000× g for 20 min, at 25°C (Centrifuge Jouan, BR4i model, France). Blanks were prepared using only the phosphate buffer 0.1 mol L^{-1}, pH 7.5.

Determination of the immobilization rate: The enzyme: support complexes were filtered through quantitative paper under vacuum and the residues were washed 3 times with 50 mL of distilled water. The filtrates and the waste waters were collected for the determination of the unabsorbed protein content, by the Lowry method (Lowry, 1951; Hartree, 1972), using bovine serum albumin as standard. The same method was used for the protein quantification in the 0.1% enzymatic solution. The amount of immobilized enzyme was obtained by the difference between the protein content of the enzymatic solution and that one found for the sum of the filtrates and waste waters. The immobilization rate was calculated by the Eq. 1:

$$IE(\%)=\frac{\text{Amount of immobilized enzyme}}{\text{Amount of added enzyme}}\times 100 \quad (1)$$

Where,

IE = Immobilized Enzyme

Determination of the operational stability of immobilized pancreatin: The reusability of pancreatin was evaluated by measuring the residual activity of the enzyme after it has been used for up to 20 times to hydrolyze whey. The quantification of Phenylalanine (Phe) exposed by the enzymatic hydrolysis of whey proteins was used for measuring the enzyme activity. As described before by the group (Morais *et al*., 2004), this corresponds to the Exposure Rate (ER) of Phe, which may be measured by Second Derivative Spectrophotometry (SDS).

First, pancreatin was immobilized, as described above, using the condition that produced the highest immobilization rate. Then, appropriate volumes of a 10% (w v^{-1}) whey solution were added to the enzyme: substrate complex in order to obtain an Enzyme:Substrate ratio (E:S) of 1%. The mixture was stirred for 1 h, at 25°C, in a velocity sufficient to keep the complex in suspension. Then, it was centrifuged at 11000× g for 20 min, at 25°C and filtered through quantitative paper under vacuum. The residues were washed 3 times with 5.0 mL of distilled water and submitted to successive hydrolysis giving rise to six hydrolysates corresponding to the 1st, 2nd, 5th, 10th, 15th and 20th utilizations of the immobilized enzyme. The hydrolysates were, then, freeze-dryer (Labconco, 77500 model, Kansas city, MI, USA) and submitted to the SDS.

Determination of exposure rate of phenylalanine: The exposure rate of phenylalanine was measured by SDS, as described before by the group (Lopes *et al*., 2005; Morais *et al*., 2004). Briefly, the absorbance of the

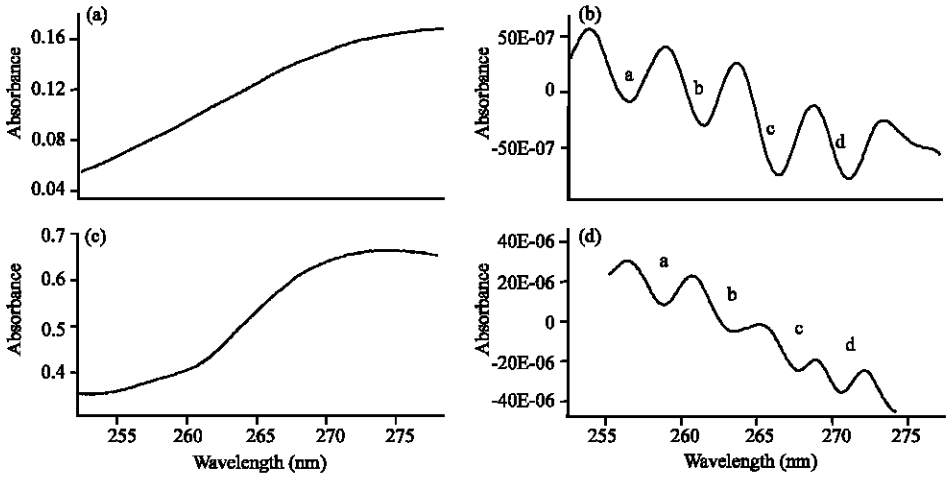

Fig. 1: Absorbance and second derivative spectra of Phe in a solution containing Tyr and Trp (A and B, respectively) and of hydrolysate obtained after the second hydrolysis (C and D, respectively) in pH 6.0

samples was measured from 250-280 nm. Second derivative spectra were drawn (CECIL spectrophotometer, CE2041 model, Buck Scientific, England) and the area of the third negative peak c (Fig. 1) was used to calculate the amount of Phe in the samples, employing a standard curve. A software GRAMS-UV (Galactic Industries Corporation, Salem, NH, EUA) was used to draw the second derivative spectra.

For the standard curve, stock solutions of Phe (6.05×10^{-4} mol L^{-1}), Tyr (5.52×10^{-4} mol L^{-1}) and Trp (4.90×10^{-4} mol L^{-1}) were prepared in 0.01 mol L^{-1} phosphate buffer, pH 6.0. Then, 10 mL of each solution were mixed and successive dilutions of this mixture were made to have Phe concentrations in a range from 0.13-1.01×10^{-4} mol L^{-1}. Spectra of these diluted solutions were recorded from 250-280 nm and the area of the third negative peak of Phe was used to draw the standard curve in function of its concentration.

Statistical analysis: All experiments were carried out in triplicate. Differences between means of areas of negative peaks were evaluated by Analysis of Variance (ANOVA) and Duncan test (Pimentel-Gomes, 2000). Differences were considered to be significant at $p<0.05$ throughout this study. The least square method was used to fit the standard curve and the adequacy of the linear model ($Y = aX + b$) was tested at $p<0.05$.

RESULTS AND DISCUSSION

Immobilization rate of pancreatin: Table 1 shows that the type of support affected the immobilization rate of pancreatin, which was much higher for the activated Carbon (96 and 100%) than for alumina (8 and 37%), for all tested conditions. This result could be explained by the fact that proteins having high structural stability called hard proteins are barely adsorbed on hydrophilic supports, such as alumina. Pancreatin could fit this category, since one of its main components, chymotrypsin is considered a hard protein (Nakanishi *et al.*, 2001).

Table 1: Immobilization rate of pancreatin on activated carbon and on alumina

	Immobilization supports	
Parameters	Activated carbon (%)*	Alumina (%)*
30 min/25°C	$100.0\pm2.0^{a/1}$	$17.0\pm5.0^{b/2}$
60 min/25°C	$96.0\pm4.0^{a/1}$	$11.0\pm1.0^{b/2}$
90 min/25°C	$96.0\pm1.0^{a/1}$	$37.0\pm3.0^{a/2}$
12 h/5°C	$99.0\pm1.0^{a/1}$	$8.0\pm1.0^{c/2}$

*Each value represents the mean of triple determination. Data presented are means±SD. Different letters are significantly different ($p<0.05$) for different treatments and the same support. Different numbers are significantly different ($p<0.05$) for different supports and submitted to the same treatment

Table 1 can observe that no significant differences were shown for the immobilization rate of pancreatin when AC was the support used. Thus, the condition of 30 min, at 25°C, was chosen for evaluating the efficiency of the hydrolysis because it employed the shortest time, which is advantageous from the economical point of view. In case of AL, the condition chosen was that of 90 min, at 25°C, since it produced the highest immobilization rate.

No report was found in the research concerning the immobilization of pancreatin on solid supports. Some researches were performed with the enzymes, which are

part of the pancreatin, such as trypsin, chymotrypsin and carboxypeptidase A. In one of these studies, trypsin was immobilized by adsorption on a polymer (Eudragit S-100, 2 and 4%) and 99% of the enzyme was linked to the support (Sardar *et al*., 1997). This result is close to that obtained in this research for pancreatin, when using AC as the support. Vanková *et al*. (2001) immobilized α-chymotrypsin from bovine pancreas on two supports containing saccharide residues and the best immobilization rate was of 30% using the α-galactosyl-polyacrilamida as support. This result was similar to just one value obtained in the present research for pancreatin with AL (90 min, 25°C) and much less inferior to those obtained here with AC. The immobilization rate of carboxypeptidase A on polyethylene terephtalate was around 70%, i.e., lower than those obtained here for pancreatin with AC (Vértesi *et al*., 1999).

SDS spectra and standard curve: The absorbance and SDS spectra of Phe in a mixture of aromatic amino acids and of the hydrolysate obtained after the second hydrolysis (pH 6.0) are shown in Fig. 1. In case of Phe (Fig. 1b), we can see 4 negative peaks, indicated by letters a, b, c and d, situated within the range of 250-280 nm with maxima at 253, 258, 263, 268 and 273 nm and minima at 257, 262, 267 and 272 nm. The SDS spectrum of the hydrolysate is close to that of Phe, with negative peaks situated in almost the same wavelengths. The likeness among the spectra of standard amino acids and proteins had previously been described by Ichikawa and Terada (1977), research with several native and denaturated proteins. The same result was previously achieved in the laboratory using different enzymes for hydrolyzing casein and skim milk (Morais *et al*., 2004; Lopes *et al*., 2005; Soares *et al*., 2006).

On the other hand in some reports in the research, the number of negative peaks for Phe was different from that found in the present research. Thus, 5 peaks for this amino acid in pH 7.0 were shown by Ichikawa and Terada (1977), while Miclo *et al*. (1995) described the presence of 6 in pH 1.9. These discrepancies could be associated to several factors such as forms of Phe (free or Esther), type of equipment (spectrophotometer and software used for measuring absorption and derivative spectra), properties of the solvent and pH used (Ichikawa and Terada, 1977; Levillain and Fompeydie, 1986).

Concerning the standard curve of Phe, the one using the area of negative peak c presented the best correlation coefficient (the highest and most significant). The curve Eq. 2 and the correlation coefficient were:

$$y = 3.0077x + 0.7587 \text{ e } R^2 = 0.9576 \quad (2)$$

This result is in agreement with others in the research by Ichikawa and Terada (1977) and Zhao *et al*. (1996) and also with previous studies carried out in the laboratory (Lopes *et al*., 2005; Soares *et al*., 2006; Morais *et al*., 2004), since in all these researches a linearity for the standard curve of Phe in presence of Tyr and Trp in several concentrations was shown.

Operational stability of immobilized pancreatin: As shown in Table 2, when pancreatin was immobilized in activated carbon, its enzymatic activity kept unchangeable (100%) up to 5 times, indicating that one could use this enzyme 5 times, using the conditions established here. It is worth stating that even after using this enzyme 20 times, its activity was 65% of the initial value, which may be considered a good one, comparing to other data of the research by Li *et al*. (2001) and Lin *et al*. (2002).

Although, Whitaker (1994) states that immobilized enzymes may be reused up to reach 50% of their initial activity in the case, it would be important to keep their activity near 100%, because the interest is associated to hydrolyze proteins in order to remove Phe and prepare dietetic supplements for phenylketonurics, since the more the protein is hydrolyzed the highest the Phe exposition and the easiest the removal of this amino acid are expected to be.

Table 2: Operational stability of immobilized pancreatin on activated carbon and alumina

	Immobilization supports			
No. of times utilization of the enzyme	Activated carbon		Alumina	
	Exposure rate of Phe*	Enzyme activity (%)	Exposure rate of Phe*	Enzyme activity (%)
1	$59\pm2^{a/2}$	100	$176\pm10^{a/1}$	100
2	$59\pm4^{a/2}$	100	$180\pm4^{a/1}$	100
5	$62\pm2^{a/2}$	100	$143\pm13^{b/1}$	82
10	$50\pm5^{b/2}$	83	$153\pm12^{b/1}$	82
15	$36\pm3^{c/2}$	65	$142\pm3^{b/1}$	82
20	$42\pm5^{c/2}$	65	$119\pm7^{c/1}$	67

*Exposure rate of Phe, in mg of Phe/100 g of hydrolysate. Each value represents the mean of triple determination. Data presented are means±standard deviation. Different letters are significantly different ($p<0.05$) for different number of times of utilization of the enzyme using the same support

The results of the pancreatin immobilization on alumina were just a little less satisfactory than on activated carbon (Table 2), since the constancy of the enzymatic activity was obtained up to 2 times (100%) and reduced to 67% after having been used for 20 times. These results show clearly that the immobilization of pancreatin on carbon activated and on alumina may be considered when the interest is associated to the use of this enzyme in large scale industrial processes.

Only one research was found in the study concerning the operational stability of a component of pancreatin. Thus, Nouaimi *et al.* (2001) reported that the immobilization of trypsin on polyester wool using bovin serum albumin as spacer was able to keep the enzyme activity for a week. However, the number of times that the enzyme could be used during this period was not mentioned.

CONCLUSION

Among the conditions tested for the immobilization of pancreatin on Alumina (AL), the one which employed a contact of 90 min at 25°C, was the most favorable giving rise an immobilization rate of 37%.

However, for the Activate Carbon (AC), no significant difference between the conditions tested was observed and the immobilization rate varied from 96-100%. In the test for hydrolyzing Whey, the AC was also better than AL, since it allowed the reuse of the enzyme by up to 5 times, while using AL the maximum was of 2 times.

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