Brazilian Journal
of Chemical
Engineering

ISSN 0104-6632
Printed in Brazil
www.abeq.org.br/bjche

Vol. 33, No. 02, pp. 251 - 260, April - June, 2016
dx.doi.org/10.1590/0104-6632.20160332s20140111

NOVEL APPLICATION OF POROUS AND CELLULAR MATERIALS FOR COVALENT IMMOBILIZATION OF PEPSIN

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(Submitted: October 14, 2014 ; Revised: March 5, 2015 ; Accepted: May 5, 2015)

Abstract - Pepsin was immobilized *via* covalent bonds on different carriers: a silica gel carrier, acrylic beads, and a cellulose-based carrier - Granocel. All carriers were functionalized through the presence of -OH, -COOH, $-NH_2$, or glycidyl groups on their surfaces. Three different cross-linkers were used for activation thereof. The results showed that Granocel activated by glutaraldehyde or carbodiimide and silica gel activated by glutaraldehyde were suitable carriers for the expression of enzyme activity. The optimum pH range for the native enzyme was 2.5-3.5 and this range was extended to the value 6.5 in the case of enzyme immobilized on the silica gel carrier and on Granocel. The optimum temperature values for the native and immobilized enzyme were in the range 37-40 °C and 40-50 °C, respectively. The activity of the immobilized pepsin at different values of pH and temperature was higher in comparison with the activity of the free enzyme.
Keywords: Covalent immobilization; Silica gel carriers; Acrylic beads; Cellulose-based carriers; Pepsin.

INTRODUCTION

Pepsin (EC 3.4.23.1) is one of the principal enzymes in the digestive system from the group of aspartic proteases. As an endopeptidase, pepsin decomposes most proteins and peptides to oligopeptides, and has milk-curdling activity (Konkular and Gunasekaram, 2002). Moreover, this enzyme is capable of efficient proteolysis, even at low temperatures (Ahn *et al*., 2012). Its potential applications in food production or biomedical industries have become more common and have replaced many traditional methods. For example, bovine or porcine pepsin is regarded as a substitute for calf rennet or is used for protein digestion prior to studies on identification of proteins as well as characterization and production of bioactive peptides (Konkular and Gunasekaram, 2002; Ticu *et al*., 2004; Shukla and Devi, 2005). Especially in the latter practical application of pepsin, immobilization is a prerequisite step that allows increasing the stability of biocatalysts in order to achieve higher profitability (Cooper *et al*., 1990, Kamatari *et al*., 2003; Gamze and Senay, 2007).

Immobilized enzymes are frequently used in various industrial and biotechnological processes (Liese and Hilterhaus, 2013) owing to their higher stability, allowing longer operation at higher temperatures, a wider pH range, high ionic strength, or the presence of toxic substances (Bahar and Celebi, 1998; Mateo *et al*., 2007). There are many known methods for enzyme immobilization, reviewed and published in a large number of papers (Liese and Hilterhaus, 2013). The most popular methods are adsorption and covalent binding; however, the most commonly used method is covalent immobilization (Esawy *et al*., 2008; Ahmed *et al*., 2013; Liese and Hilterhaus, 2013). The reasons for the covalent stabilization of enzymes by immobilization can be found in the mul-

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tipoint and multisubunit attachment of enzymes to surfaces, generation of a favourable microenvironment (e.g., promoting the partitioning of some deleterious compounds), and in the fact that they can also prevent intermolecular interactions (e.g., contact with the gas/liquid interface, aggregation, hydrolysis by proteolytic enzymes) (Liese and Hilterhaus, 2013). Studies on the immobilization of pepsin have been conducted for a long time. One of the invaluable advantages of immobilization of proteases on an insoluble matrix is prevention of enzyme autolysis. Currently, immobilized pepsin is mainly used for fragmentation of antibodies in the biomedical industry (generation of $F(ab')_2$ from IgG) or in strictly specific digestion of proteins which are soluble in an acid medium (Rea and Ultee, 1993). Although there are several commercial preparations of immobilized pepsin (e.g. immobilized on agarose beads by Thermo Scientific - Pierce or immobilized on poly(styrene divinylbenzene) particles by Life Technologies - Applied Biosystems), there is no systematic study on the immobilization of this enzyme on a broader spectrum of carriers with different porosity and with different functional groups on their surface. So far, pepsin has been immobilized on different types of carriers using different methods of immobilization. For example, Stigter *et al.* (2008) described the use of pepsin microreactors consisting of pepsin immobilized in a fused-silica capillary. Pepsin was also immobilized on chitosan beads (Altun and Cetinus, 2007), acrylic copolymers with methacrylates as a co-monomer (Li *et al*., 2004; Shukla and Devi, 2005; Hu *et al*., 2006), a modified alumina complex (Ticu *et al*., 2005), agarose beads (Kurimoto *et al*., 2001), silica gels (Line *et al*., 1971), and cellulose-based carriers (Dumitriu *et al*., 1987). Most researchers focused their attention on one carrier (Hu *et al*., 2006) or a limited number of carriers but with the same basic structure (Poojari *et al*., 2009). Moreover, a comparative study on the preparations obtained is very difficult as the authors used different analytical methods and different substrates such as casein (Dharmapuri and Saiprakash, 1999), hemoglobin (Anson, 1938), and albumin (Gray and Billings, 1983).

The main goal of our work was to bind pepsin on a range of carriers *via* various anchor groups to find the matrix and its functionality that is preferred by pepsin (Figure 1). For this reason, pepsin from hog stomach was covalently immobilized on acrylic beads (a cheap, synthetic, and non-biodegradable carrier with a medium pore diameter – d_m= 9.4 nm), silica gel carrier (an expensive carrier inert for the microenvironment with a narrow pore diameter – d_m= 4.0-4.6 nm), and a cellulose-based carrier (a moderately expensive, biodegradable carrier with wide pores with size exclusion limit 10^5-10^6 Da). These materials were selected because they possess -glycidyl, -NH_2, -COOH, and/or –OH groups on their surface and could be activated with different cross-linkers (none, glutaraldehyde, carbodiimide, and divinylsulfone, respectively). The activity of immobilized pepsin at different values of pH and temperature was studied with special attention devoted to extension of pH profiles that would allow specific digestion of proteins soluble not only in an acidic environment. Finally, the reusability of the pepsin-carrier preparations obtained in a batch reactor is also discussed.

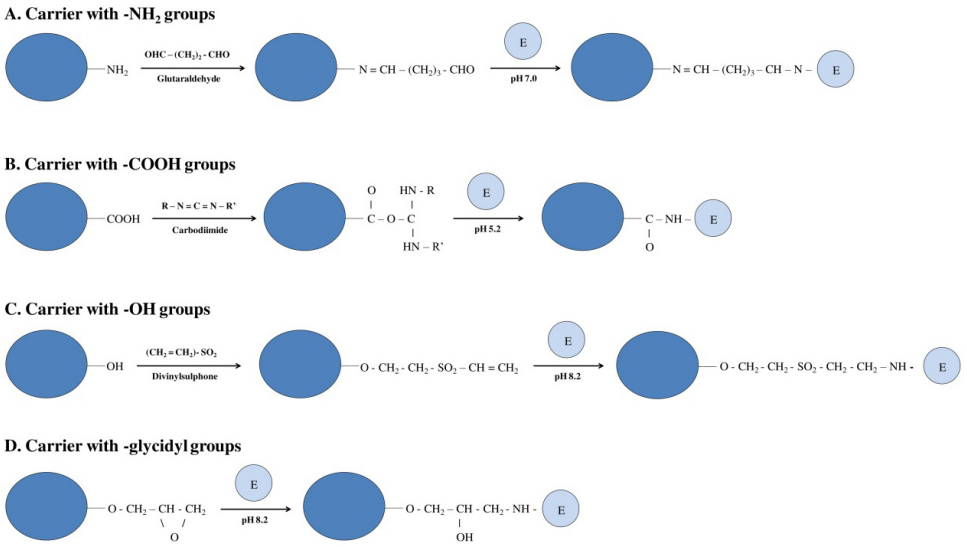

Figure 1: Scheme of enzyme immobilization processes on carriers possessing various functional groups activated by (A) glutaraldehyde, (B) carbodiimide, (C) divinylsulphone or (D) without any activator, E – enzyme.

MATERIALS AND METHODS

Materials

Tris(hydroxymethyl)aminomethane (Tris), glutaraldehyde (GLA), divinylsulfone (DVS), (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (CDI), Folin & Ciocalteu's phenol reagent, bovine serum albumin, (3-glycidoxypropyl)triethoxysilane (GPTS), 3-aminopropyltriethoxysilane (APTS), and 2-aminoethyl-3-aminopropylmethyl-dimethoxysilane (AEAPMDS) were purchased from Sigma-Aldrich (Germany). Pentaethylenehexamine and pepsin (EC 3.4.23.1) were provided by Fluka (Germany). The other reagents, all of analytical grade, were supplied by POCh (Poland).

Carriers

The acrylic carrier (A; carriers 1-3), composed of butyl acrylate and ethylene glycol dimethacrylate, was modified by reaction with ethylenediamine in toluene (Bryjak, 2003). It should be emphasised that the aminolysis in a large excess of ethylenediamine led to formation of *N*-amidoamine (A-NH_2) and a stoichiometric amount of hydroxyl groups (A-OH) (Bryjak, 2005). Simultaneously, carboxylic groups appeared in the polymer network (A-COOH) as a result of hydrolysis of the ethylene glycol dimethacrylate copolymer. The carrier was selected due to its very high capability of immobilization of other enzymes such as penicillin acylase (Bryjak *et al*., 1993), amylases (Line *et al*., 1971), trypsin (Bryjak *et al*., 2008), and laccase (Bryjak *et al*., 2007). It was donated by Tarchomin Pharmaceutical Plant (Poland).

The medium mesoporous silica gel matrix, Kieselgel 60 (K; carriers 4-7), was purchased from Degussa (Essen, Germany). The gel surface was modified by direct grafting of organosilanes (GPTS in the case of K1-glyc, AEAPMDS in the case of K2-NH_2 and K3-NH_2, and APTS in the case of K4-NH_2) as described previously (Jarzębski *et al*., 2007). The Kieselgel 60 matrix was selected due to its better properties than those of other silica gels with a narrower diameter of pores (IE Int. Enzymes silica gel) tested for casein hydrolysis with immobilized trypsin (Jarzębski *et al*., 2007). Moreover, this matrix was very good for laccase immobilization (Rekuć *et al*., 2010).

The cellulose-based carrier Granocel (G; carriers 8-12) was obtained as described previously (Serys *et al*., 1994; Bryjak *et al*., 2007). For the experiments, two basic Granocel matrices were selected: Granocel-500 (G1 with a size exclusion limit of 10^5 Da) and Granocel-2000 (G2 with a size exclusion limit of 10^6 Da). NH_2-functionalized G1 was prepared using pentaethylenehexamine as a long spacer arm (G1-NH_2), whereas G1-COOH was obtained according to the procedure described previously (Bryjak *et al*., 2007). Unmodified G2-OH was the third basic carrier. It must be pointed out that carriers G1 have –OH groups as well. The Granocel matrix was selected as a material with a very good potential for immobilization of other enzymes: tyrosinase (Labus *et al*., 2011), laccase (Bryjak *et al*., 2007), or invertase and trypsin (Bryjak *et al*., 2008).

The basic properties of the tested carriers are summarised in Table 1.

Table 1: Structural parameters of acrylic (A), silica gels (K1 - K4) and cellulose-based carriers (G1 - G2), activators of functional groups (DVS - divinyl sulfone, GLA - glutaraldehyde, CDI - carbodiimide) and pH values of the coupling mixtures (Labus *et al*., 2011; Zynek *et al*., 2011).

Type of carrier	Main parameters of carrier	Carrier number	Symbol of carrier	Functionality *used for activation*	Activator/pH
Acrylic carriers	Granules 0.2 ~ 0.3 mm S_{BET} 690 m^2/g V_p 0.8 cm^3/g d_m 9.4 nm	 1 2 3	 A-NH_2 A-COOH A-OH	 -NH_2 -COOH -OH	 GLA/7.0 CDI/5.2 DVS/8.2
Silica gels	Granules 0.2 ~ 0.5 mm S_{BET} 270 ~ 302 m^2/g V_p 0.52 ~ 0.57 cm^3/g d_m 4.0 ~ 4.6 nm	4 5 6 7	K1-glycidyl K2-NH_2 K3-NH_2 K4-NH_2	- $(-NH_2)_2$ $(-NH_2)_2$ -NH_2	-/8.2 GLA/7.0 GLA/7.0 GLA/7.0
Cellulose based carriers	Particle size 0.16 – 0.32 mm SV 1.7 mL/g; WR 2.6 g/g; SEL 10^5 Da	8 9 10 11	G1-NH_2 G1-NH_2 G1-COOH G1-COOH	-NH_2 -OH -COOH -OH	GLA/7.0 DVS/8.2 CDI/5.2 DVS/8.2
	Particle size 0.16 – 0.32 mm SV 2.1 mL/g; WR 6.5 g/g; SEL 10^6 Da	12	G2-OH	-OH	DVS/8.2

Abbreviations: S_{BET} - specific surface area, V_P - mesopore volume, d - pore sizes, SV - specific volume, WR - water retention, SEL - size exclusion limit (for dextran)

Immobilization of Pepsin

To have better insight into the efficiency of immobilization based on the reaction of different anchor groups, the following protocol was completed. Each group of carriers has a fixed superstructure and different types of functional groups: -glycidyl, -COOH, -OH, and -NH_2. The procedures of immobilization were selected to be applied mainly to protein amine groups during enzyme-carrier coupling. However, this can be performed at different values of pH (see Fig. 1). The functionalized carrier (5 mL) was washed with distilled water and then with a buffer appropriate to the kind of anchor groups. 5 mL of functionalized carrier contained 9 g of acrylic carriers (A), or 7 g of Granocel (G) or 12 g of silica gel (K). The activation of the carriers and the process of enzyme immobilization were carried out as described earlier (Bryjak *et al*., 2007). The activators (DVS, GLA, CDI) and the pH values of the enzyme solution used for immobilization are provided in Table 1. The activated carrier was suspended in a pepsin solution containing 3 mg/mL of protein (5 mL of the enzyme solution per 5 mL of the carrier). The unbonded protein was removed by washing with the following buffers with different pH and ionic strength values: 0.1 M phosphate buffer pH 7.0, 0.5 M NaCl, 0.1 M phosphate-citrate buffer pH 3.5, and distilled water. The active groups remaining on the carriers were blocked by washing with 0.5 M Tris-HCl buffer (pH 5.0). The obtained enzyme-carrier preparations were intensively rinsed with 0.1 M phosphate buffer (pH 3.5), then were stored in 0.1 M phosphate-citrate buffer, pH 3.5, at 4 °C, and shortly prior to the experiments were washed with this buffer several times.

All the eluates with unbound proteins were collected and analysed for the presence of proteins and for measurement of proteolytic activity. The amount of bound proteins was calculated from the difference between the amount of protein used for immobilization and the amount washed off during the immobilization process. Protein yields were calculated as a ratio of the amount of bound proteins to the concentration of protein in the solution of enzyme used for the immobilization process. Activity yield was calculated as a ratio of the activity of immobilized pepsin to the activity of native pepsin in solution used for the immobilization process. These yields were expressed as a percentage.

Pepsin Activity Assay

The native pepsin activity was determined by the Anson method with some modification (Anson, 1938). The activity of native enzyme (0.5 mL) was measured in the presence of 0.5 mL 1% bovine albumin in 0.1 M phosphate-citrate buffer (pH 3.5). The reaction mixture was incubated for 1 hour at 37 °C and after this time the reaction was stopped by addition of 2 mL of 5% trichloroacetic acid (TCA), and the denaturated protein was centrifuged (10 min, 15 000 rpm). The absorbance of the digested product in the supernatant was measured spectrophotometrically at 280 nm (Varian Cary 50 Bio, Germany). In the control samples, TCA was added before incubation of the substrate and enzyme. The proteolytic activity unit (AU) was defined as the amount of the enzyme (native or immobilized) that yielded a rise in absorbance of 0.001 per minute of incubation with the substrate under the assay conditions. The protein concentration was determined by the Lowry method, using bovine serum albumin as a standard (Lowry *et al*., 1951).

The activity of the immobilized enzyme was measured in a well-mixed (150 rpm) thermostated (37 °C) batch reactor. The immobilized enzyme (0.25 mL) was suspended in 5 mL of 0.1 M phosphate-citrate buffer pH 3.5 and placed into the reactor. Next, 5 mL of preheated 1% bovine albumin in 0.1 M phosphate-citrate buffer pH 3.5 was added. After 1 hour of incubation, 1 mL of the sample was taken out and added to the stopping solution (1 mL of TCA). After centrifugation (10 min, 15 000 rpm), the absorbance of the released product was determined spectrophotometrically.

Effect of Temperature and pH Values on Native and Immobilized Enzyme Activity

The effect of different pH values on the native and immobilized biocatalyst activities was studied at 37 °C using 0.1 M phosphate-citrate buffer with a pH range from 2.5 to 8.0. The optimum temperature was determined by incubation of the samples in a temperature range of 20-60 °C. The other reaction conditions were the same as in the activity assay.

Operational Stability of Immobilized Pepsin in a Batch Reactor

The operational stability of the selected preparations in the thermostated batch reactor was assessed by incubating a 0.25-mL sample (diluted in 5 mL of 0.1 M phosphate-citrate buffer pH 3.5) with 5 mL of 1% bovine albumin in the same buffer at 37 °C with moderate mixing (150 rpm). After 1 hour of incubation, 1 mL of the sample was taken out and added to 1 mL of 5% TCA (stopping solution). Released oligopeptides were determined spectrophotometri-

cally at 280 nm after centrifugation (10 min, 15 000 rpm). The immobilized enzyme was collected by filtration, washed five times with the buffer, and suspended in a new solution of the substrate to begin the next cycle of activity measurement.

Statistical Analysis

All the results are expressed as mean ± SD from three experiments (n=3). The data were analysed using one-way ANOVA followed by a post hoc Tukey test. Values of $P \leq 0.05$ only were reported as a statistically significant.

RESULTS AND DISCUSSION

Proper selection of the immobilization carriers has a significant impact on the quality and efficiency of enzyme binding. A suitable carrier should have a proper structure characterized by the presence of suitable functional groups, an applicable chemical and mechanical strength, easy regeneration, and low manufacturing costs.

Enzyme Immobilization

The usefulness of carriers for enzyme immobilization can be evaluated on the basis of four main parameters: the amount of bound protein, enzyme activity, specific activity of bound protein, and storage stability. According to the common procedures, pepsin was immobilized on activated matrices and the results obtained are shown in Table 2.

In the first stage of this study, we examined the ability of pepsin to bind to the acrylic carriers (carriers 1-3) containing three different functional groups (-OH, -NH_2, and -COOH). These types of carriers were activated for the immobilization process by the addition of divinylsulphone (DVS), glutaraldehyde (GLA), or carbodiimide (CDI), respectively. The schematic enzyme immobilization used in this work is presented in Fig.1. Since pepsin loses its activity at high pH values, the enzyme preparation was dissolved in solutions with pH 3.5 and the activity assay was performed. Then, depending on the crosslinking agent and immobilization techniques used, the enzyme preparation was adjusted to the appropriate value of pH of the reaction mixture. It was observed that pepsin immobilized on the acrylic supports *via* the -OH and -NH_2 activator groups had no enzymatic activity (Table 2), in spite of the confirmed binding of the enzymatic protein on the surface of this type of carrier. In the case of GLA (carrier 1) there was a 51.9% yield of protein bound to the carrier and in the case of DVS (carrier 3) 35.1% of protein yield. It can be assumed that amino acids responsible for enzymatic activity are involved in the enzyme binding on the surface of different carriers. In the case of pepsin, carboxylic groups (-COOH) are very important, as they were not blocked only in the case of the acrylic carrier activated by CDI (carrier 2) and the activity of the bound enzyme was 39.4 AU/mL of the carrier and the protein yield was 27.9%. For comparison, Poojari *et al*. (2009) obtained 23% protein yield during pepsin immobilization on silicone elastomers. Additionally, pepsin immobilized by the -COOH groups of the acrylic supports showed very good stability during one month of storage (about 44.7%) compared to the native enzyme (Table 2).

Table 2: Efficiency and storage stability of pepsin immobilized on the tested carriers.

Symbol of carrier	Carrier number	Bound protein [mg/mL]	Protein yield [%]	Measured activity [AU/mL carrier]	Activity yield [%]	Specific activity [AU/mg of protein]	Activity after one month of storage [%]
Native enzyme	-					1991.3	0
Acrylic carriers							
A-NH_2	1	1.73 ±0.30	51.98	0	0	0	-
A-COOH	2	0.88 ±0.07	27.93	39.4±1.89	7.9	44.8	44.7
A-OH	3	1.08 ±0.10	35.18	0	0	0	0
Silica gels							
K1-glycidyl	4	0.46 ±0.11	15.05	0	0	0	-
K2- NH_2	5	1.65 ±0.16	49.55	78.0 ±0.72	15.9	47.3	40.5
K3- NH_2	6	1.70 ±0.27	50.98	44.3 ±1.10	8.9	26.1	39.8
K4- NH_2	7	2.74 ±0.05	82.26	12.0 ±0.53	3.0	4.4	69.2
Cellulose-based carriers							
G1-NH_2	8	0.83 ±0.15	24.96	55.3 ±0.42	11.2	66.6	27.2
G1-NH_2	9	1.84 ±0.09	59.71	12.0 ±0.66	2.4	6.5	93.9
G1-COOH	10	0.90 ±0.16	29.04	109.6 ±0.93	22.1	121.8	16.4
G1-COOH	11	0.98 ±0.20	31.86	28.0 ±0.44	5.6	28.6	62.9
G2-OH	12	1.29 ±0.08	42.02	0	0	0	-

[a] Activity measured in the presence of albumin as substrate. The enzyme activity unit (AU) was defined as the change of absorbance of 0.001 due to the action of the native or immobilized enzyme during 1 minute under the assay conditions.

The second group of tested carriers were cellulose-based supports, Granocel, having a different specific volume and water content (carriers 8-12). It was shown that carrier G1, characterized by a lower specific volume (SV = 1.7 mL/g) and water retention (WR = 2.6 g/g), exhibited more effective binding of pepsin. The best activity yield of immobilized pepsin (22.1%) was obtained for Granocel activated by carbodiimide (carrier 10) comprising -COOH groups on its surface (Table 2). Substantially lower activity of pepsin was achieved after activation of the supports by glutaraldehyde (11.2%) and divinylsulphone (from 2.4 to 5.6%). In the case of G2 (carrier 12), a carrier with high water retention (WR = 6.5 g/g), no proteolytic activity of immobilized pepsin was demonstrated. Similar to the acrylic carriers, the cellulose-based support Granocel showed different binding capacity depending on the pH value of the immobilization reaction. The best activity of immobilized pepsin (109.6 ± 0.93 AU/mL of carrier) was observed for the preparation obtained under acidic conditions of immobilization. The activity of the bound enzyme decreased with increasing pH value of the reaction medium. The results are not surprising if we consider that the optimum pH value for native pepsin is an acidic pH in the range 2-3.

In our study, we also tested classical silica gel supports (carriers 4-7) functionalized by attachment of glycidyl (K1) or amino groups (K2-NH_2, K3-NH_2, K4-NH_2) and activated by glutaraldehyde. The results presented in Table 2 show that the silica gel matrix with active amino groups allowed binding of pepsin with activity yields from 8.9 to 15.9% and protein yields amounting to 50% (carriers 5-7).

Taking into account that pepsin acts on large substrates (e.g., albumin), the glutaraldehyde method should offer the best preparations due to its tendency to form dimeric or trimeric structures, resulting in the formation of a long spacer arm. In contrast, the lowest effectiveness should be expected for carbodiimide activation or by bonding directly *via* glycidyl residues. In this case, the enzyme is bound directly onto the carrier surface. Thus, the effect of activation *via* DVS could give intermediate results. However, this expectation does not match the results obtained. The longest spacer arm can be formed in the case of carrier no. 8, which was functionalized with pentaethylenehexamine. Moreover, the worst results should be obtained for carriers no. 2, 4 and 11, which was not the case.

The use of carriers with a defined pore size is important in obtaining efficient immobilization yield. It is assumed that, with increasing diameter of the pore, the surface area is increased, which allows better access of protein to this surface during enzyme immobilization (Zynek *et al*., 2011). In our studies, we did not observe this relationship. The silica gel support with an associated pore diameter (d_m = ~4.0-4.6 nm) immobilized pepsin better than the acrylic support with a large pore diameter (d_m = 9.4 nm).

Interestingly, the same carriers were used for immobilization of trypsin, but the effects of the immobilization were much different. Jarzębski *et al*. (2007) achieved similar amounts of protein bound to the carrier in the case of silica gel support (33.8-74.7%), but trypsin practically lost its proteolytic activity after the immobilization process. In our earlier work trypsin was immobilized very effectively onto acrylic and cellulose-based supports (Bryjak *et al*., 2008). Trypsin immobilized on the acrylic carrier activated with carbodiimide was also almost entirely inactivated (carrier 2) and immobilization *via* the –OH and –NH_2 groups was more effective (carriers 1 and 2). The yield of bound protein ranged here from 7 to 65%, but the immobilization efficiency was not satisfying (the highest amount was 26.5%). The best results were obtained for the cellulose-based carrier. The yield of bounded protein for the carrier activated by divinylsulfone was relatively high (33.1-49.9%) and the immobilization efficiency achieved was the highest (97%).

These results indicate that the immobilization conditions must be chosen strictly and carefully for each biocatalyst. Pepsin and trypsin are two proteins with proteolytic activities, but they differ in their requirements for optimal environments in the biocatalysis process. These features have a potent influence on the immobilization process as we obtained completely different results while using the same carriers.

Effect of Temperature and pH Values on Native and Immobilized Enzyme Activity

Taking into account the optimum pH value (pH 3.5) suggested by the enzyme supplier and the fact that most enzymes are stable at their pH_{opt}, it was expected that the immobilization at pH 5.2 *via* carbodiimide activation (carriers 2 and 10) would yield preparations with the highest activity. On the other hand, immobilization *via* DVS (carriers 3, 9, 11, and 12) or with glycidyl functionality (carrier 4), both at pH 8.0, would be the worst. This expectation is confirmed by the results obtained: carrier 2 is the only active sample of the acrylic matrix, whereas carrier 10 is the most active among the Granocel carriers and, generally, among all the carriers tested. Moreover, among the five carriers with the lowest expected activity, three were inactive and two had low activity.

We can conclude that, in terms of the effectiveness of pepsin immobilization, the pH value of the coupling mixture is a crucial point.

In order to compare the properties of the immobilized pepsin with those of the native enzyme, the effects of temperature and pH values on the enzymatic activities were examined. The effects of temperature on the activity of the native and immobilized pepsin in the temperature range of 20-60 °C are presented in Fig. 2 A and B. The optimum temperature values for the native and immobilized pepsin were found in the range from 37 to 40 °C and from 40 to 50 °C, respectively. The results are similar to results described for pepsin immobilized on chitosan beads, silicone elastomers, and on an aldehyde-modified polymethacrylate monolith (Altun and Cetinus, 2007, Poojari *et al*, 2009; Han *et al*., 2014), where the optimum temperature for the immobilized pepsin was higher than that for native enzyme and depended on the tested support. The increase in optimum temperature is caused by the change of the physical and chemical properties of the enzyme by the immobilization process.

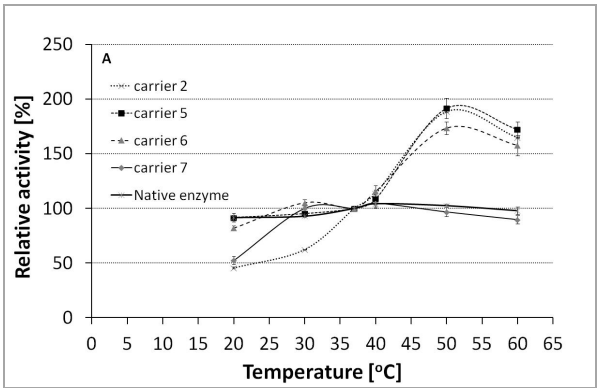

Figure 2: Activity under various conditions of temperature for native enzyme and pepsin immobilized on silica gel (carriers 5-7) and acrylic support (carrier 2) (Fig. 2 A) or cellulose-based support (carriers 8-11) (Fig. 2 B). The value at 37 °C was set arbitrarily as 100%.

The covalent bond formation *via* amino groups of the immobilized enzyme might also have reduced the conformational flexibility, as immobilized enzymes need a higher temperature to form the proper conformation to recognize and bind substrate molecule (Hu *et al*., 2006; Altun and Cetinus, 2007). In the case of pepsin, the immobilization process probably gave rise to increased rigidity of the tertiary structure of the enzyme, which prevented the molecule from expanding (Mateo *et al*., 2007). These physico-chemical changes in the structure of the immobilized enzyme can explain the thermal activity shift.

The activities of the native and immobilized pepsin at various pH values are presented in Figs. 3 A and B and a pH-dependent activity shift to higher pH values in the case of immobilized pepsin was observed. Enzyme immobilized on all tested carriers (silica gel, acrylic support, cellulose-based support) exhibited higher activities than native enzymes in the range of pH values from 3.5 to 6.5. In terms of chemical structure, pepsin is a single polypeptide chain with a molecular weight of 35 kDa. The inactive form of pepsin, pepsinogen, has 44 amino acids more than the native enzyme. The enzyme active site contains two aspartate residues lying on both sides of the water molecule. The enzyme is activated when one of the aspartate groups is ionized and the other occurs in the unionized form. These interactions make pepsin active in an acidic environment with optimum pH between the values of 1.5 and 2.0 and inactive at pH 6.5 and above. In our study, the optimum pH values for native pepsin were in the range of 2.5-3.5. The pepsin preparation immobilized on the silica gel support exhibited a pH optimum in the range of 2.5-6.5 for the K4-NH_2 carrier (carrier 7) and 4.5-6.5 for the K2-NH_2 (carrier 5) and K3-NH_2 (carrier 6) carriers. Similarly, in the case of the cellulose-based supports, we observed that immobilized pepsin did not lose its activity in the pH range from 2.5 to 6.5. At pH 6.5 the native enzyme was totally inactive. Altum and Cetinus (2007) reported that pepsin immobilized on chitosan beads had no activity at pH 7.0, while Li *et al.* (2012) reported that pepsin immobilized in a sol-gel system had no activity at pH 6.5. In the case of pepsin binding to silicone elastomers, it was observed that immobilized pepsin retained 70% of its original activity at pH 7.0 (Anson, 1938). This is probably due to reduced conformational flexibility after immobilization. Additionally, the interactions (ionic, polar, or hydrogen bonding) between the enzyme molecule and the carrier microspheres can change the ionisation of immobilized enzyme molecules (Hu *et al*., 2006). During the immobilization process different groups of the carrier react with

amine groups in pepsin, including the N-terminus, but these interactions do not disturb the proteolytic activity of pepsin.

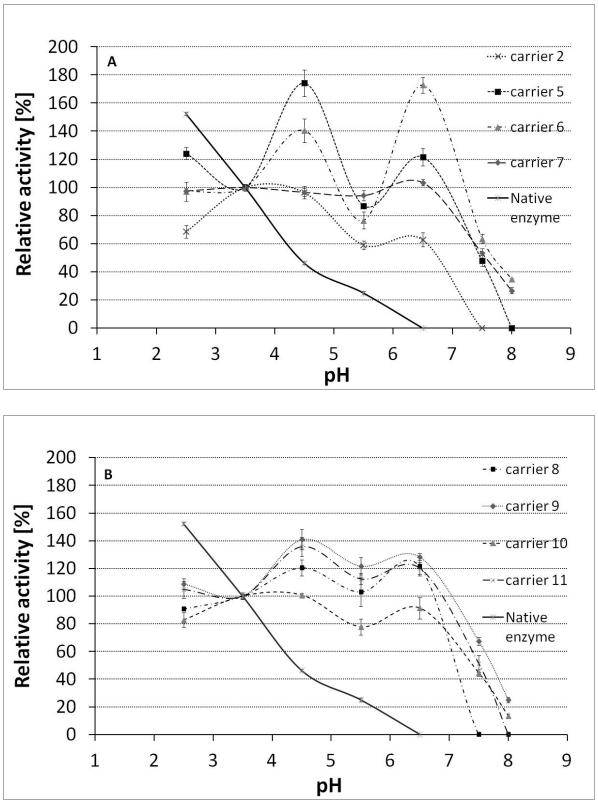

Figure 3: Activity under various conditions of pH for native enzyme and pepsin immobilized on silica gel (carriers 5-7) and acrylic support(carrier 2) (Fig. 3A) and cellulose-based support (carriers 8-11) (Fig. 3 B). The value of pH 3.5 was set arbitrarily as 100%.

The results obtained confirm that inorganic supports have greater dimensional stability than organic ones. The process of pepsin immobilization can increase the scope of its activity over a broader range of pH values in comparison to the native enzyme. Furthermore, the immobilization process probably resulted in formation of two different enzyme forms with different optima for temperature and the value of pH (Fig. 2A, B; Fig. 3A, B), which may be very suitable for industrial processes. This feature may facilitate using immobilized pepsin in the digestion of proteins soluble in slightly alkaline solutions.

Operational Stability of Immobilized Pepsin in a Batch Reactor

The operational stability, which specifies the time after which half of the initial activity of enzyme is lost, is the basic technological parameter allowing evaluation of the desirability of applying the chosen immobilization method. This parameter is very important for preparative and industrial use of different form of enzymes. In our study, the operational stability of the immobilized pepsin was tested in consecutive batch processes (Fig. 4 and Fig. 5). Among the tested immobilization carriers, the best results were obtained for silica gel (carrier 7) activated by glutaraldehyde, which retained 70% of the immobilized pepsin activity after three cycles and for Granocel activated by glutaraldehyde (carrier 8), which retained 40% or by carbodiimide (carrier 10) which retained 43% activity of the immobilized enzyme.

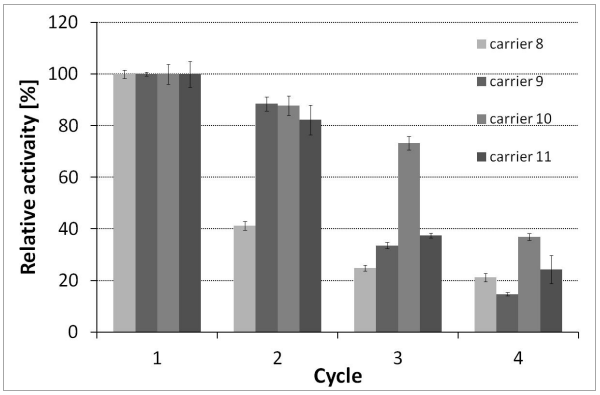

Figure 4: Operational stability for pepsin immobilized on cellulose-based carriers.

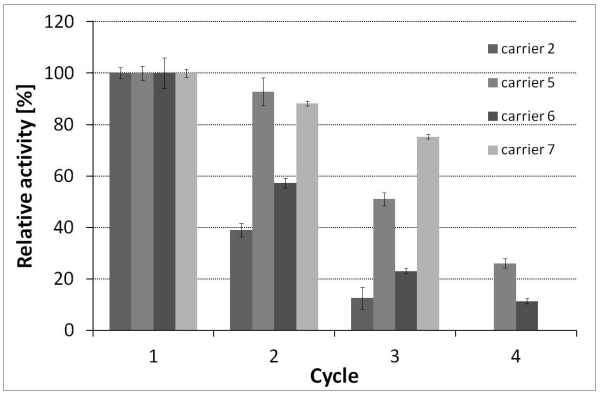

Figure 5: Operational stability for pepsin immobilized on silica-gel (carriers 5-7) and acrylic carriers (carrier 2).

Altun and Cetinus (2007) observed that pepsin immobilized on chitosan beads retained 95% of the specific activity after three cycles and this activity decreased gradually during the operational process. In the case of pepsin immobilized on acrylamide/2-hydroxyethyl methacrylate copolymer the immobilized enzyme retained 55% of its activity after six

cycles of application (Shukla and Devi, 2005). The ability of immobilized pepsin to maintain the enzymatic activity for several operating cycles gives it advantages over native, non-immobilized enzyme.

CONCLUSIONS

Three different groups of carriers (acrylic, cellulose-based, and silicagel supports) were tested for immobilization of pepsin from hog stomach. The results showed that only the cellulose-based carriers activated by glutaraldehyde or carbodiimide and silicagel activated by glutaraldehyde may be used to bind pepsin effectively. Very significant improvement in the stability of the immobilized pepsin over a broader pH range was observed, in comparison to the native enzyme. Promising results for thermal and operational stability of the immobilized pepsin were also shown, compared to the free enzyme. The results obtained are promising due to the possibility of industrial application of immobilized pepsin.

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