การจำแนกคุณลักษณะของเอนไซม์ทริปซินที่ผ่านการตรึงจากเครื่องในปลากะพง

Biochemical Characteristics of Immobilized Trypsin from Porgy (*Stenotomus chrysops*) Viscera

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Abstract: Partially purified trypsin from porgy (*Stenotomus chrysops*) viscera was immobilized onto Octyl Sepharose CL-4B. The optimal temperature and pH for the hydrolysis of *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (DL-BAPNA) by the immobilized trypsin were 55°C and 8, respectively. The immobilized trypsin exhibited a much broader pH range (6-11), temperature (20°C-80°C) activity and stability versus the free form of the enzyme. The immobilized enzyme was less sensitive to inhibition by the soybean trypsin inhibitor. According to the results, the immobilized trypsin and free enzyme retained 70.59% and 20.10% of their activity, respectively, when they were incubated with 1 μM of the soybean trypsin inhibitor. For the reusability study, the immobilized trypsin maintained 41.31% of its activity after 6 periods of activity, indicating that the immobilized trypsin had appropriate stability and could be reused.

Keywords: Immobilization, characterization, trypsin, viscera

บทคัดย่อ: เอนไซม์ทริปซินที่ผ่านการทำบริสุทธิ์บางส่วนจากเครื่องในปลากะพงสามารถถูกตรึงบน Octyl Sepharose CL-4B เอนไซม์ทริปซินตรึงรูปมีอุณหภูมิและพีเอชที่เหมาะสมสาหรับการย่อยสลาย Nα-benzoyl-DL-arginine-p-nitroanilide (DL-BAPNA) เท่ากับ 55 องศาเซลเซียสและ 8 ตามลาดับ เอนไซม์ทริปซินตรึงรูปแสดงกิจกรรมและความคงตัวต่อพีเอช (พีเอช 6-11) อุณหภูมิ (20-80 องศาเซลซียส) ในช่วงกว้างกว่าเมื่อเปรียบเทียบกับเอนไซม์ในรูปอิสระ เอนไซม์ตรึงรูปมีความไวลดลงต่อสารยับยั้งเอนไซม์ทริปซินจากถั่วเหลือง สอดคล้องกับผลการทดลองพบว่า เอนไซม์ตรึงรูปและเอนไซม์ในรูปอิสระคงกิจกรรมร้อยละ 70.59 และ 20.10 ตามลำดับ เมื่อบ่มด้วยสารยับยั้งเอนไซม์ทริปซินจากถั่วเหลืองที่ระดับความเข้มข้น 1 ไมโครโมลาร์ สำหรับการศึกษาการนำเอนไซม์กลับมาใช้ได้ใหม่พบว่า เอนไซม์ทริปซินตรึงรูปยังคงมีกิจกรรมร้อยละ 41.31 หลังจากผ่านการนำไปใช้ 6 ครั้ง แสดงว่าเอนไซม์ทริปซินตรึงรูปมีความคงตังและสามารถนำกลับมาใช้ใหม่ได้

คำสำคัญ: การตรึง การจำแนกคุณลักษณะ ทริปซิน เครื่องใน

Introduction

Trypsin is one of the major digestive enzymes, which acts on peptide bonds on the carboxyl side of lysine and arginine residues. Trypsin has a catalytic triad of serine, histidine and aspartate (Bougatef, 2013). The enzyme has many biomedical and industrial applications due to its narrow specificity that allows for more controlled proteolysis (Klomklao *et al*., 2007). Among all trypsins, fish trypsins are of immense interest because some of them are more active catalysts at relatively low temperatures compared with similar enzymes from thermophilic organisms from mammalian and plant sources, making them very suitable for a number of biotechnological and food processing applications (Simpson, 2000).

Nevertheless, the enzymes have some limitations that may hinder their industrial applications. For example, widespread use of the free form of the enzyme in batch reactions is limited by stability, recovery, and reusability (Aryee and Simpson, 2012). To enhance the convenience and cost-effectiveness of enzymes in catalysis, immobilization, among other strategies, has been suggested (Garcia-Galan *et al.*, 2011). Immobilization involves the attachment of the soluble/free enzyme onto inert solid support materials. This technique does not only fix enzyme molecules to make them reusable, but also has potential to modify the properties of enzymes, such as the stability and catalytic properties to enhance the feasibility of applications (Vidinha *et al.*, 2006). The main advantages of enzyme immobilization include reusability, ease of process control, reduced costs, and ease of separation of the enzyme from the reaction products after the desired transformation is achieved (Wu *et al*., 2005).

Certain trypsins have previously been immobilized by cross- linking, covalent attachment, entrapment in mesoporous materials, and surface adsorption (Sun *et al.*, 2015) using various supports such as controlled pore glass (Li *et al*., 2013), silica-based materials (Sun *et al*., 2015), polyamidoamine dendrimers (Wu *et al.*, 2018), chitosan (Sun *et al.*, 2017), magnetite nanoparticles (Atacan *et al.*, 2017), alginate (Ganachaud *et al.*, 2013) and lignocelluloses (Bassan *et al*., 2016). Recently, the immobilization of trypsin from Nile tilapia was reported by Azevedo *et al.* (2018). Nevertheless, the prospects of immobilizing alternative and underutilized sources of trypsin such as from fishery resources have received little attention. Therefore, the aims of this study were to investigate reaction conditions for the immobilization and physicochemical properties of immobilized porgy trypsin. To prepare the enzyme for possible commercial application, the advantages of increased operational stability as well as ease of enzyme recovery for repeated use via immobilization were also explored in this study. The studies reported here could provide useful information for using immobilized fish trypsin in industrial applications.

Materials and Methods

2.1 Reagents

Octyl Sepharose CL- 4B, N- **α**-benzoyl- DL- arginine- *p*- nitroanilide (DL-BAPNA), dimethyl sulfoxide, and glutaraldehyde were purchased from Sigma-Aldrich (Oakville, ON, Canada). Citric acid, glycine, sodium acetate, hydrochloric acid, calcium chloride, sodium hydroxide, monobasic/ dibasic potassium phosphate and monobasic/ dibasic sodium phosphate were purchased from Fisher Chemicals (Nepean, ON, Canada). All solvents used in this study were purchased from Fisher Scientific (Whitby, ON, Canada) except ethanol which was obtained from Commercial Alcohols (Boucherville, QC, Canada).

2.2. Sample preparation

Porgy (*S. chrysops*) were purchased from a local market in Montreal (Waldman Plus). After capture, the fish were chilled in ice and off- loaded approximately 10 h thereafter. Fish were bought immediately when the fish arrived at the market. Fish were kept in ice with the sample/ ice ratio of 1: 2 (w/ w) and transported to the research laboratory within 30 min. The fish were eviscerated and the viscera were removed. The collected viscera were cut into pieces with a thickness of 1-1.5 cm. The viscera was defatted with acetone according to the

method of Klomklao *et al*. (2007) and stored at –20°C.

2.3. Preparation of crude extract

Defatted viscera powder was stirred continuously in 50 mM Tris– HCl, pH 7. 5 containing 5 mM $CaCl_2$ at a ratio of 1:10 (w/v) at 4 °C for 3 h. The homogenate was centrifuged for 30 min at 4 °C at 10,000 g using a Beckman J2-21 refrigerated centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA) to remove the tissue debris. The supernatant obtained was designated as the " crude extract".

2.4 Trypsin partial purification

Trypsin from the viscera of porgy was partially purified using ammonium precipitation. Purification processes were carried out in a cold room (4 °C). During purification, protein concentration was measured at 280 nm, and trypsin activity was assayed with DL- BAPNA as a substrate (Erlanger *et al*., 1961).

The crude enzyme extract was mixed with Brij 35 to obtain a final concentration of 0. 2%, held overnight and finally centrifuged at 10,000 g for 30 min. The supernatant obtained was subjected to ammonium sulfate precipitation and the fraction precipitating between 40 and 60% saturation was collected. The pellet obtained after centrifugation at 10,000 g for 30 min was dissolved in 15 mL of 50 mM Tris–HCl, pH 7. 5 containing 5 mM $CaCl_2$ and dialyzed against

20 volumes of 50 mM Tris– HCl, pH 7. 5 containing 5 mM $CaCl_2$ overnight with three changes of the dialysis buffer and then concentrated by lyophilization. The powdered partially purified porgy trypsin thus obtained, was used for immobilization studies.

2. 5 Porgy trypsin immobilization on octyl Sepharose CL-4B

The porgy trypsin immobilized onto octyl Sepharose CL- 4B was prepared according to the method of Aryee and Simpson (2012). The previously washed octyl Sepharose CL- 4B equilibrated with 10 mM sodium phosphate buffer, pH 7 was added to 40 mg/mL enzyme solution in 10 mM sodium phosphate buffer, pH 7 (1: 10 w/ v resin to buffer) , and incubated on Multi- purpose rotator (Barnstead International, Iowa, USA) at 50 rpm and 4°C for 6 h. The trypsin- resin mixture was vacuum- filtered using a Pyrex® Büchner funnel with a sintered (fritted) glass disc, and the filtrate was collected. The immobilized porgy trypsin was washed with 3 changes of 10 mL 10 mM sodium phosphate buffer, pH 7. The immobilized trypsin was then filtered and vacuum- dried in a desiccator over silica overnight. The dried immobilized trypsin from porgy viscera was stored at -20°C.

2. 6 Activity determination of free and immobilized trypsin

The activities of the free trypsins were determined using DL- BAPNA as substrate following the method of Erlanger *et al*. (1961). An aliquot of the enzyme solution (200 μL) was added to 1000 μL of reaction mixtures containing 800 μL of 1 mM DL-BAPNA solution in 0.05 M Tris-HCl buffer (pH 8.2, containing 0.02 M $CaCl_2$) and 200 μL of 0.05 M Tris-HCl buffer containing 0.02 M $CaCl_2$, pH 8.2. The mixture was incubated at room temperature (25°C) for precisely 10 min. The release of *p*-nitroaniline from DL-BAPNA was measured at 410 nm using a DU® 800 UV-vis spectrophotometer (Backman-Coulter, Brea, Canada).

For the immobilized trypsin, the method was modified as follows: about 10 mg of dry immobilized albacore tuna trypsin were added to 800 μL of 1 mM DL-BAPNA in 0.05 M Tris-HCl buffer (pH 8.2, containing 0.02 M $CaCl_2$) and 400 μL of 0.05 M Tris-HCl buffer containing 0. 02 M $CaCl_2$, pH 8. 2. After incubation at room temperature (25°C) for 10 min, the reaction mixture was centrifuged at 12,000 g (accuSpin Micro 17R, Fisher Scientific, Massachusetts, USA) for 1 min at room temperature (25°C) and the absorbance of the supernatant was measured at 410 nm in the DU® 800 UV-vis spectrophotometer (Backman-Coulter, Brea, Canada). One unit of trypsin activity was defined as 1 μmol *p*-nitroaniline released/ min, using 8800 M^{-1} cm^{-1} as the extinction coefficient of *p*-nitroaniline at 410 nm (Erlanger *et al*., 1961).

2.7 Characterization of free and immobilized porgy trypsins

2.7.1 pH and temperature profile of free and immobilized porgy trypsins

Activity of free and immobilized porgy trypsins were assayed over the pH range of 3.0-11.0 (10 mM citric acid-NaOH buffer for pHs 3.0-3.5; 10 mM potassium phosphate buffer for pHs 6.0-8.5; 10 mM glycine-NaOH buffer for pHs 9.0-10.0 and disodium hydrogen phosphate-NaOH buffer for pH 11) at room temperature (25°C) for 10 min.

For temperature profile study, the activities of free and immobilized porgy trypsins were assayed at different temperatures ranging from 20 to 70°C for 10 min at pH 8.2. The relative trypsin activity (%) was calculated using the highest trypsin activity obtained as 100%.

2. 7. 2 pH and thermal stability of free and immobilized porgy trypsins

The effect of pH on free porgy trypsin stability was evaluated by measuring the residual activity after incubation enzyme

in various buffers (pHs 4, 5, 6, 7, 8, 9, 10 and 11) for 30 min at room temperature (25°C). For the immobilized enzyme, 10 mg of the immobilized trypsin were incubated at the various pHs under study (4, 5, 6, 7, 8, 9, 10 and 11) for 30 min at room temperature (25°C). At the end of the incubation period, the sample was centrifuged at 3,000g (accuSpin Micro 17R, Fisher Scientific, Massachusetts, USA) at room temperature (25°C) for 5 min and the supernatant (buffer) was carefully removed. To the damp immobilized albacore tuna trypsin was added the substrate (1 mM DL-BAPNA in 0.05 M Tris-HCl buffer, pH 8.2, containing 0.02 M $CaCl_2$) and assayed as described above. The relative trypsin activity (%) was calculated using the highest trypsin activity obtained as 100%.

For thermal stability studies, the free and immobilized porgy trypsins were incubated at different temperatures (20, 30, 40, 50, 60, 70, and 80°C) for 30 min in a temperature controlled water bath (Shaking Water Bath 25, Precision Scientific, Chicago, USA). Thereafter, the treated samples were suddenly cooled in iced water. The residual activities of free and immobilized porgy trypsin were assayed as described earlier using 1 mM DL-BAPNA (in 0.05 M Tris-HCl buffer, pH 8.2, containing 0.02 M $CaCl_2$) as substrate at room temperature for 10 min. The relative trypsin activity (%) was calculated using the highest trypsin activity obtained as 100%.

2. 7. 3 Effect of soybean trypsin inhibitor on free and immobilized porgy trypsins

The effect of soybean trypsin inhibitor on the activities of the free and immobilized porgy trypsin was evaluated. Soybean trypsin inhibitor was added into the standard reaction assay to obtain final concentrations of 0, 0.25, 0.50, 0.75 and 1 μM. The remaining activities of free and immobilized porgy trypsins were determined using DL- BAPNA as substrate at room temperature (25°C) and pH 8.2 for 10 min, as mentioned above. The residual trypsin activity (%) was calculated using the trypsin activity without the inhibitor as 100%.

2. 7. 4 Reusability of immobilized porgy trypsin

The reusability of immobilized porgy trypsin was determined during six repeated batches (10 min/batch) of the hydrolysis of DL-BAPNA at room temperature (25°C). Each assay was carried out as mentioned above. Between consecutive batches, immobilized porgy trypsin was separated and washed twice with 10 mM potassium phosphate buffer for pH 7. Residual activity was determined and compared with the activity of the first batch. The relative trypsin activity

(%) was calculated using trypsin activity of the first batch as 100%.

2.8 Statistical analysis

All experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

Results and Discussion

The comparison of the pre- and post-self-assessments

3.1 Optimum pH for the hydrolyses of DL-BAPNA by free vs immobilized porgy trypsins

The effect of pH on the activity of immobilized porgy trypsin onto Octyl Sepharose CL-4B and the free form on DL-BAPNA hydrolysis is shown in Figure 1a. Optimal hydrolyses were obtained at pHs 8 and 8.5 for the free enzyme and the immobilized enzyme, respectively. Thus, the optimum pH for the hydrolysis of the substrate by the immobilized trypsin shifted to a more alkaline pH compared to that of the free form. This shift toward a more alkaline region could be possibly due to secondary interactions (e.g., ionic and polar interactions, hydrogen bonding) between the enzyme and polymeric support (Bayramoglu *et al.*, 2008). When the enzyme was immobilized, the negative charge was carried on the surface of the support. After the formation of immobilized enzyme, the negatively charged support could increase the density of protons around the active site of the immobilized enzyme. Therefore, the pH within the bulk solution was higher than that of immobilized enzyme. To compensate for this effect, the optimum pH of the immobilized enzyme had to shift to a higher pH (Kang *et al.*, 2005). Bayramoglu *et al.* (2008) reported that there was a shift in the optimal pH of the immobilized trypsin onto poly(GMA-MMA)-g-MAA beads to a higher value (9.5) when compared to that of the free trypsin. Moreover, the pH activity profile of the immobilized porgy trypsin onto Octyl Sepharose CL-4B was broader than that of the free trypsin (Figure 1a). This indicates that the free enzyme is more sensitive to pH changes, and immobilization can preserve the enzyme activity over a wider range of pH. Similar results have been reported for immobilized trypsin and other proteases onto polyaniline (Purcena *et al.*, 2009) and sterilized cotton gauze bandage (Seabra and Gil, 2007). The possible cause of this phenomenon is that the interactions between the support and enzyme could stabilize the conformation of the active site

of the enzyme. Therefore, the immobilized enzyme could keep relatively higher activity at a wider pH range. This improvement of property of immobilized trypsin could enhance the feasibility of its potential application in various industries.

3.2 Optimal temperatures for the hydrolyses of DL-BAPNA by free vs immobilized porgy trypsins

Figure 1b shows the optimal temperatures for DL-BAPNA hydrolyses by the free form versus the Octyl Sepharose CL-4B immobilized porgy trypsins. The free form of the enzyme had an optimal temperature of 40ºC, while its immobilized counterpart had an optimal temperature of 55ºC, using the same substrate for hydrolysis. Moreover, the immobilized enzyme could keep relatively high activity over a wider temperature range than that of the free form. The result suggests that the immobilization made the enzyme sturdier than its free form. Similar results have been observed for trypsin immobilized to other supports like polyaniline (Purcena *et al*. , 2009) and hydrophobic cellulose- coated silica nanoparticles (Sun *et al*., 2015). The increase in optimum temperature of immobilized enzyme was caused by the changing physical and chemical properties of the enzyme. The non- covalent multipoint interactions via amino groups of the enzyme might also reduce the conformational flexibility for the binding to its substrate (Sears and Clark, 1993).

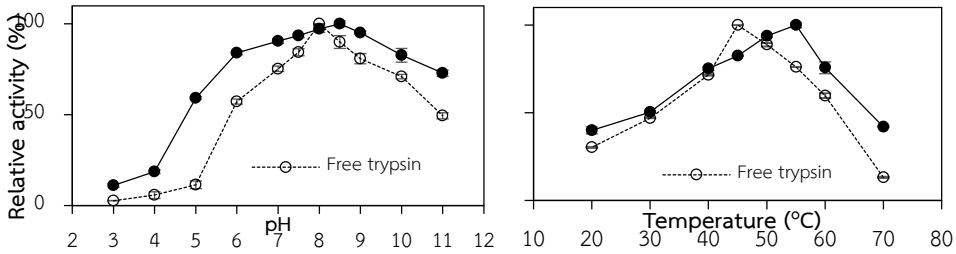


Figure 1. pH (a) and temperature (b) profiles of immobilized porgy trypsin onto Octyl Sepharose CL-4B and the free form. Bars represent the standard deviation (*n*=3)**.**

3.3 pH and thermal stabilities of the free versus the immobilized porgy trypsins

The pH stabilities of the free versus the Octyl Sepharose CL- 4B immobilized porgy trypsins are presented in Figure 2a. Both the free and immobilized porgy trypsins were stable over a broad pH range (pH 6-11). Nonetheless, the immobilized enzyme was less susceptible to activity loss at extreme pH values (both acid and alkaline) versus the free form. Kang *et al*. (2005) reported that immobilized trypsin onto poly[(methyl

methacrylate)-co-(ethyl acrylate)-co-(acrylic acid)] latex particles was more resistant to both acidic and alkaline media than the free form. The results presented above indicate that immobilized trypsin was less sensitive to pH than its free form, and that the stability of the enzyme against pH was significantly improved by immobilization.

Thermal stabilities of both the free and Octyl Sepharose CL- 4B immobilized porgy trypsins were also evaluated using DL-BAPNA hydrolysis to measure residual activities (Figure 2b). After incubation of free and immobilized enzymes at different ten

show that the inactivation rate of the immobilized porgy trypsin onto Octyl Sepharose CL-4B was much slower than that free trypsin (Figure 2b). The higher stability of immobilized enzyme could partly be caused by the limitation of autolysis (Bayramoglu *et al.*, 2008). Additionally, the increased thermostability of Octyl Sepharose CL- 4B porgy immobilized trypsin can also be ascribed to improved conformational stabilization by a stronger degree of attachment of the enzyme on the support (Octyl Sepharose CL- 4B) through hydrophobic interactions.

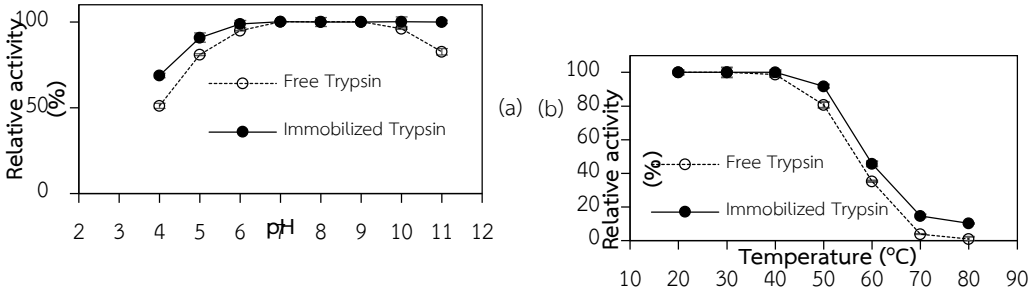
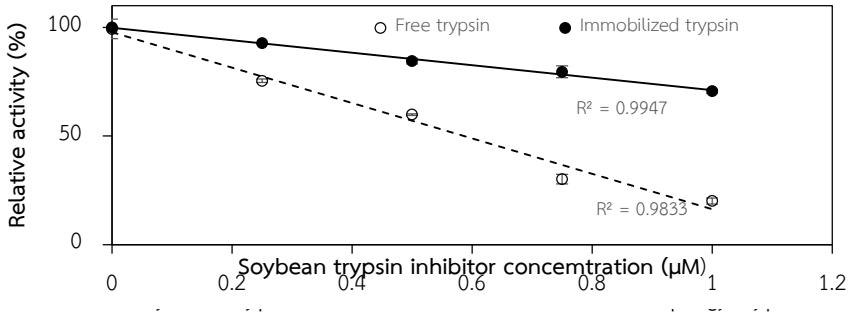


Figure 2. pH (a) and thermal (b) stabilities of free and immobilized porgy trypsin onto Octyl Sepharose CL-4B. Bars represent the standard deviation (*n*=3)

3.4 Effect of soybean trypsin inhibitor on free and immobilized porgy trypsin

Soybean trypsin inhibitor (SBTI) is one of the well-known trypsin inhibitors. It is a plant protein which has inhibitory activity against serine proteinases such as trypsin. The residual activities of the free and the Octyl Sepharose CL-4B immobilized porgy trypsins subjected to inhibition by SBTI are shown in Figure 3, which indicates that the free porgy trypsin was more sensitive to the inhibitor than the immobilized porgy trypsin. At a level of 0.25-1 μM SBTI, the free porgy trypsin lost more activity than the immobilized porgy trypsin at the same concentration of inhibitor solution with the value ranging from 17.31% to 50.49%. The result suggests that the Octyl Sepharose CL-

4B immobilized porgy trypsin was inhibited to a lesser degree by soybean trypsin inhibitor than the free form. Shtelzer *et al*. (1992) also reported that the sol-gel-entrapped trypsin activity was stable when they were incubated with soybean trypsin inhibitor. The less sensitivity of the immobilized enzyme to SBTI inhibition may be due to the active sites of free enzyme molecules were relatively more accessible to the inhibitor molecules, to enable more binding and more obstruction of substrate binding and its transformation to products. On the other hand, the active sites of the immobilized enzyme molecules were shielded by the support partially or completely, so the inhibitor molecules could not occupy the active sites of the enzyme as much to achieve the same degree of substrate binding and transformation. When mixed with the substrate molecules, the size of substrate molecules (BAPNA, molecular weight: 138 Da) was much smaller than that of soybean trypsin inhibitor (molecular weight: 20,100 Da) (Koide and Ikenaka, 1973), thus the substrate molecules could easily diffuse and gain access to the active site and cause the enzymatic reaction. From the above, it is obvious that the steric hindrance caused by the support material might protect the immobilized enzyme molecules from the inhibition. This feature of the immobilized enzyme could permit its use in certain applications such as modification of complex raw materials with naturally present inhibitors instead of the free form of the enzyme.



Figur Octyl Sepharose CL-4B.

3. 5 Reusability of immobilized porgy trypsin onto Octyl Sepharose CL-4B

The reusability of immobilized enzymes is important for economical use of an enzyme. To demonstrate the advantage of immobilized trypsin on supports, a reusability of immobilized porgy trypsin onto Octyl Sepharose CL- 4B was evaluated. As

shown in Figure 4, the relative activity of immobilized albacore tuna trypsin onto Octyl Sepharose CL-4B decreased gradually as the cycle number increased. The marked activity retention displayed by immobilized albacore tuna trypsin during the first three cycles implied that minimal desorption and activity loss of enzyme from the support (Octyl Sepharose CL- 4B) occurred on repeated use. This can be attributed to the strong affinity between the support and the enzyme produced by the immobilization procedure. This also indicates that trypsin from porgy remained adsorbed on the Octyl Sepharose CL-4B after incubation with the substrate during repeated use, while the free enzyme could not be reused after the first cycle because of difficulty in separation. The activity of immobilized trypsin decreased after reusability. This may have occurred due to the protein diffusional limitations imposed by the considerably high amount of deposits that the reaction product accumulated on the matrix surfaces. As a consequence, it caused mass transfer restrictions to the protein molecules. However, these diffusion restrictions are not imposed on reactants and other products (Lage *et al*., 2016). Aryee and Simpson (2012) reported that immobilized lipase from grey mullet (*Mugil cephalus*) viscera retained 32% of its initial activity after the sixth cycles. The reusability of the immobilized enzyme is one of the most important advantages of enzyme immobilization. Immobilization was an effective way to produce a heterogeneous system which enabled easy separation and recovery from the reaction media for repetitive use. The capacity to reuse the enzyme will decrease the cost of a relatively expensive catalyst.

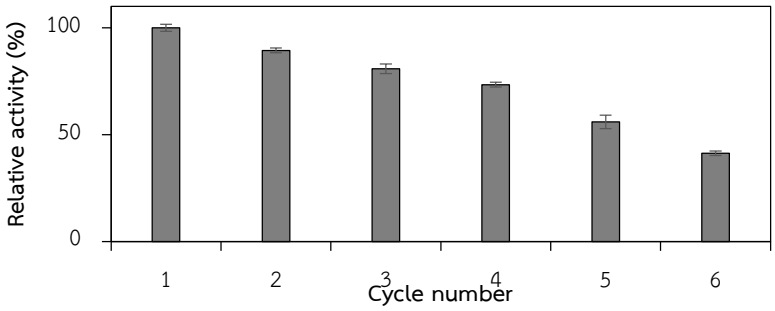


Figure 4. Ope IB. Activities are relative to the cycle showing the highest activity. Bars represent the standard deviation (*n*=3)**.** Different letters on the bars indicate significant differences (*P*<0.05)

Conclusion

Immobilized trypsin from porgy viscera had different biochemical properties compared to its free form. The optimum pH and temperature of immobilized enzyme were higher than those of its free counterpart. The immobilized porgy trypsin onto Octyl Sepharosr CL- 4B also showed higher pH and thermal stabilities than its free form. Additionally, immobilization reduced the sensitivity of the enzyme to inhibition by soybean trypsin inhibitor. This study demonstrated the simplicity of the separation and immobilization protocols together with the high activity of immobilized albacore tuna trypsin during repeated use. Therefore, immobilized trypsin from porgy viscera can be potential novel immobilized enzymes for future industrial applications.

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