Technical Sciences, 2019, 22(1), 35–43

UNIWERSYTET WARMIŃSKO-MAZURSKI W OLSZTYNIE

Quarterly peer-reviewed scientific journal
ISSN 1505-4675
e-ISSN 2083-4527
TECHNICAL SCIENCES
Homepage: www.uwm.edu.pl/techsci/

APPLICATION OF MODIFIED SILICA GEL IN THE PROCESS OF TRYPSIN IMMOBILIZATION

Justyna Miłek, Sylwia Kwiatkowska-Marks, Ilona Trawczyńska

Department of Chemical and Bioprocess Engineering
Faculty of Chemical Technology and Engineering
University of Technology and Life Sciences

Received 27 February 2018, accepted 8 January 2019, available online 14 January 2019.

Key words: trypsin, immobilization, silica gel, biocatalyst.

Abstract

The paper presents the use of modified silica gel for the production of immobilized trypsin from bovine pancreas. Silica gel was modified with 3-aminopropyltriethoxysilane, followed by glutaraldehyde. The influence of stirring time on activity of the prepared biocatalyst was determined for individual stages of the modification. Activity of both native and immobilized trypsin was measured using Kunitz method. At the temperature of 55°C and pH 7.6 native and immobilized trypsin onto modified silica gel indicate optimum activity. The influence of multiple recycling and storage time on activity of immobilized trypsin was tested. After fourteen days of storage at the temperature of 4°C immobilized trypsin exhibits 75% of its initial activity.

Correspondence: Justyna Miłek, Zakład Inżynierii Chemicznej i Bioprocesowej, Wydział Technologii i Inżynierii Chemicznej, Uniwersytet Technologiczno-Przyrodniczy im. Jana i Jędrzeja Śniadeckich, ul. Seminaryjna 3, 85-326 Bydgoszcz, phone: 52 374 90 49, e-mail: jmilek@utp.edu.pl

Introduction

Trypsin (EC 3.4.21.4) is one of the most important digestive enzymes. Optimal pH for enzyme activity ranges between 7 and 9. The role of trypsin is hydrolysis of proteins. It causes certain peptide bonds to break, decreasing the number of allergenic proteins in hypoallergenic food production (MOHAMAD et al. 2015). Trypsin is also used to improve texture of fish products and tenderness of meat; to stabilize meat; for tissue culture (cleavage of proteins); for extraction of seasonings and flavorings from vegetable and animal proteins (GOMEZ, ROMERO 2009)

Application of native enzymes has following disadvantages: influence of environmental conditions on enzyme deactivation; difficulty in removing a substrate from solution which may lead to contamination of the product. For this reason, enzyme immobilization on carriers may significantly contribute to cost reduction and improvement of the process, due to the reusability of the same portion (*Endopeptidases: Advances in Research…* 2012).

Silica gel is among carriers used for immobilization enzymes (GOMEZ, ROMERO 2009, SUN et al. 2015). It is characterized by high mechanical strength, low cost and modifiability. Additionally, techniques of trypsin immobilization onto a carrier with the use of covalent bonds become increasingly common. Such method consists of forming a covalent bond between functional groups of the carrier and protein. This ensures that enzyme is rigidly attached to the surface of a carrier. Immobilization using such method involves several stages. Firstly, activation of the carrier is conducted by attaching a reactive group, then the enzyme attaches (YANG et al. 2010). Cross-linking agents can be glutaraldehyde and 3-aminopropyltriethoxysilane (SHEN et al. 2011).

Cellulose was also used as a trypsin immobilization carrier (*Methods of Enzymatic Analysis* 2012). However, cellulose tends to be washed away in aqueous solutions during hydrolysis. Thus, silica gel was found to be a more effective carrier. Silica gel does not swell in aqueous environment, exhibits a decent mechanical strength, and is able to undergo a heat treatment. SHEN et al. (2011) presented that surface modification of silica nanoparticle by aminopropyl groups (3-aminopropyltriethoxysilane) causes an increase in adsorption ability of bovine serum albumin (BSA), in comparison to unmodified silica nanoparticles. YANG et al. (2010) proved that activity of immobilized lipase on aminosilica gel activated by glutaraldehyde is greater compared to immobilized lipase without being activated by glutaraldehyde. Thus, modification of the gel has been carried out, using 3-aminopropyltriethoxysilane (3-APTES) and glutaraldehyde solutions. Figure 1 illustrates the process of trypsin immobilization on silica gel with the use of 3-aminopropyltriethoxysilane (3-APTES) and glutaraldehyde.

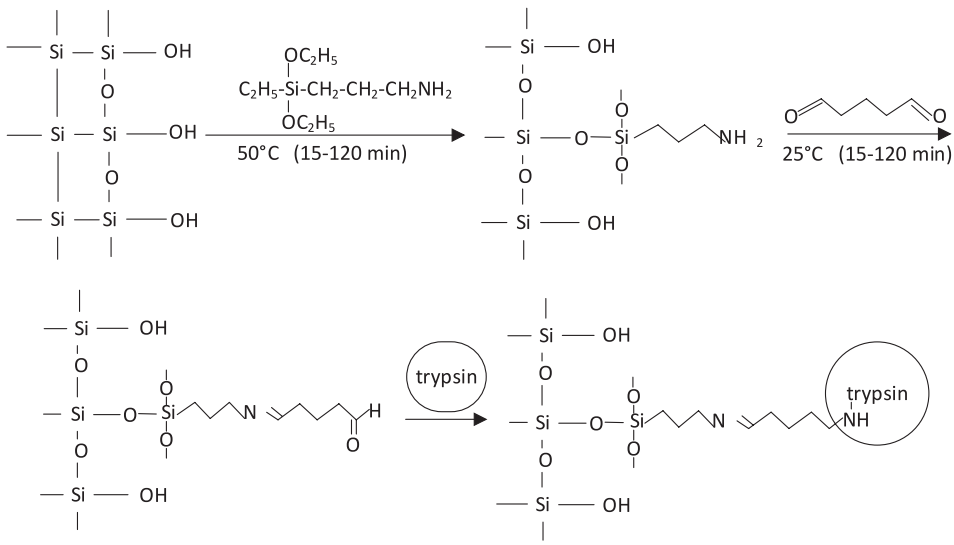

Fig. 1. The process scheme of trypsin immobilization with the use of 3-APTES and glutaraldehyde

Major and most important source of protein in milk is casein (2.4-2.6%). Hydrolysis of proteins with the use of enzymes occurs in significantly milder temperature conditions. Due to selectivity of enzymes, the number of by products produced in hydrolyzed proteins is limited.

The aim of the study was to produce immobilized trypsin from bovine pancreas on modified silica gel in optimal conditions. Modification of silica gel by 3-aminopropyltriethoxysilane (3-APTES) and glutaraldehyde in immobilization process was applied for the first time. Activity of native and immobilized enzyme was measured using Kunitz's method (ZHOU et al. 2011, SALAR et al. 2017). Effects of temperature within the range of 35-75℃ and pH between 3 and 9 on activity of native and immobilized trypsin on modified silica gel were determined. Reusability of the biocatalyst was also tested.

Materials and Methods

For the enzyme assays, native enzymes or immobilized trypsin carrying the same amount of enzyme were used. In this study, all experiments were done triplicate, and the results are expressed as mean ± SD.

Activity of native and immobilized trypsin

Trypsin enzyme activity was measured using modified Kunitz method. The reaction mixture was prepared as 0.2 ml enzyme solution or three biocatalyst (0.9-1 g) and 2 ml 1% casein in 0.1 M phosphate buffer (pH 7.6). After incubation at 55°C for 20 min, 3 ml trichloroacetic acid solution was added to terminate the reaction. After that, the mixture was incubated at room temperature for 10 min. Samples were centrifuged at 12,000 rpm for 10 min. The absorbance values were measured spectrophotometrically at 280 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmol tyrosine per minute under the assay conditions. Activity retention for the immobilized trypsin was determined by the ratio between the activity of the immobilized trypsin and the activity of a similar amount of the free enzyme. Relative activity trypsin was determined according to equation 1:

$$\text{Relative activity} = (A/A_{\text{max.}})\cdot 100\% \quad (1)$$

where:

A – the measured activity trypsin,

$A_{\text{max.}}$ – the activity of trypsin in optimum conditions.

Relative activity was also presented by CARAMORI et al. (2010), MONTEIRO and SILVA (2007), SUN et al. (2015), YANG et al. (2010) for activity immobilized and native trypsin.

Process of trypsin immobilization

10 g of silica gel was weighed and added to 50 ml 10% 3-aminopropyltriethoxysilane solution. Mixture was being stirred at temperature of 50°C for specified time. The influence of stirring time (15 min, 30 min, 60 min, 120 min) on immobilized trypsin activity was tested. Modified 3-APTES silica gel was washed by 0.1 M phosphate buffer (pH 7.6). Then, silica gel was stirred with 25 ml 2.5% glutaraldehyde solution at 25°C. The influence of stirring time with glutaraldehyde within the range of 15–120 min on activity of immobilized enzyme was tested. Such modified silica gel was added to 20 ml of native trypsin solution with a concentration of 0.3 mg/ml. The effects of stirring time within the range of 15–120 min at temperature of 25°C of activated silica gel with native trypsin solution on immobilized trypsin activity were tested.

The effect of temperature and pH on activity of native and immobilized trypsin

The optimum temperature was determined by incubating native and immobilized trypsin 0.1 M phosphate buffer pH 7.6 at temperatures in the range 35–75°C for 20 min. At the end of the time, 3 ml trichloroacetic acid solution was added to terminate the reaction and the enzyme activity of each sample was determined.

The effect of pH on trypsin activity was measured at 55°C over a range of pH 3.0–9.0. For pH 3.0 a citric acid-sodium dihydrogen phosphate buffer was used, and between pH 6.0 and 9.0, the activity was determined in 0.1 M phosphate buffer. To analyze the effect of pH on trypsin stability, the enzyme solutions were incubated in buffers (0.2 ml enzyme solution or three biocatalyst) of varying pH values (3.0–9.0) for 20 min at 55°C. Residual activity was determined under optimum conditions (phosphate buffer, pH 7.6, 55°C).

Repeated uses and storage stability of immobilized trypsin

Hydrolysis of casein by immobilized trypsin was carried out at temperature of 55℃ and pH equal to 7.6. After incubation at 55℃ for 20 min, activity was determined, and immobilized trypsin on silica gel was placed into 1% casein solution.

The storage stability at the temperature of 4℃ on activity of immobilized trypsin was tested. Biocatalyst was stored for 14 days. Measurements were taken at temperature of 55℃ and pH equal to 7.6. Hydrolysis of casein was carried out on the day of immobilized trypsin production as well as after 1, 3, 7, 10 and 14 days of storage.

Results and discussion

This study aimed to optimize production of immobilized trypsin from bovine pancreas on silica gel. Table 1 presents the effect of activation time of silica gel by 10% 3-APTES solution on immobilized trypsin activity. Extension of stirring time from 15 min to 120 min causes a 40% increase in enzyme activity.

The effect of activation time of silica gel by 2.5% glutaraldehyde solution on immobilized trypsin activity is presented in Table 1. Extension of stirring time to 120 min causes a 20% increase in enzyme activity.

The effect of activation time by trypsin solution on activity was also tested (Tab. 1), in order to optimize the production of immobilized trypsin.

Table 1

Relative activity of immobilization trypsin at different activation time

Activity of immobilization trypsin [%]				
Activation time	15 min	30 min	60 min	120 min
I step – 10% 3-APTES				
	62	70	87	100
II step – 2.5% glutaraldehyde				
	81	85	88	100
III step – solution of trypsin				
	81	83	88	100

The temperature profile of free and immobilized trypsin was determined by incubating trypsin with casein solution at different temperatures (35–70°C). Figure 2 presents the effect of temperature on activity of tested native and immobilized trypsin. The optimal temperatures for both native and immobilized trypsin were similar (55°C for both).

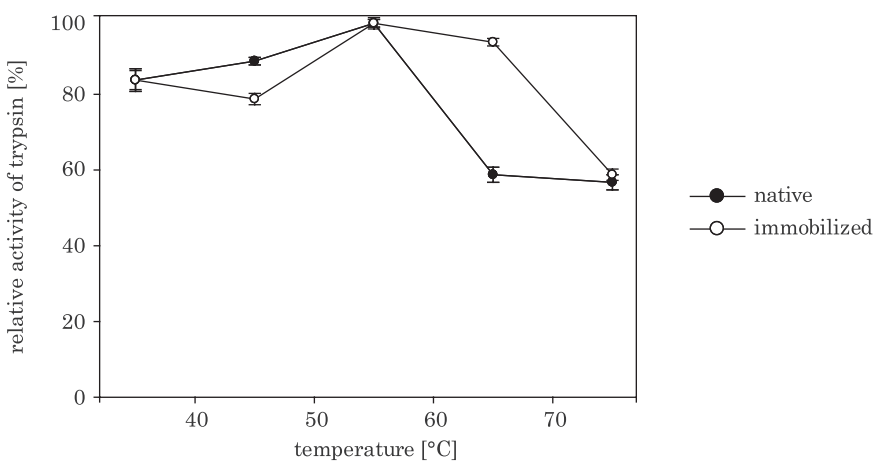

Fig. 2. The effect of temperature on activity of native and immobilized trypsin (t=20 min, pH 7.6)

The relative activities of free and immobilized enzyme at different temperatures were normalized to that of 55°C. At the temperature of 65°C immobilized trypsin activity is more than 35% greater that activity of native trypsin, suggesting that biocatalyst is more tolerant to inactivation at high temperature. Studies influence of temperature on immobilized trypsin activity are comparable to the results achieved by MONTEIRO and SILVA (2007). Those experimentals for

temperature within the range of 25°C to 65°C indicate that immobilized trypsin on silica at 65°C possesses higher activity values, compared to the activity of native trypsin. This proves that biocatalyst obtained through the immobilization process is more resistant to temperature conditions.

The variation of pH in the reaction medium can affect the stability of the enzyme and, consequently, its activity. The effects of reaction pH on the activities of immobilized trypsin were studied and compared with those for the free trypsin. Activity of immobilized and native trypsin for pH between 3 and 9 during hydrolysis of milk proteins was assayed. As can be seen, both free and immobilized trypsin had their highest activities at pH 7.6, which is the "optimum pH" value of trypsin (Fig. 3). In the whole of the pH range studied the immobilized trypsin maintained its activity better than the native trypsin. Immobilized trypsin at pH 3 and 9 exhibited greater activity compared to native trypsin, by 30% and over 20% respectively. Possibly, the covalent interaction between the silica gel-(3-APTES)-glutaraldehyde nanoparticles and trypsin increased the rigidity of the trypsin molecules, inhibiting the extensional distortion and nonspecific aggregation of the trypsin molecules, thus allowing them to better resist the effect of pH change.

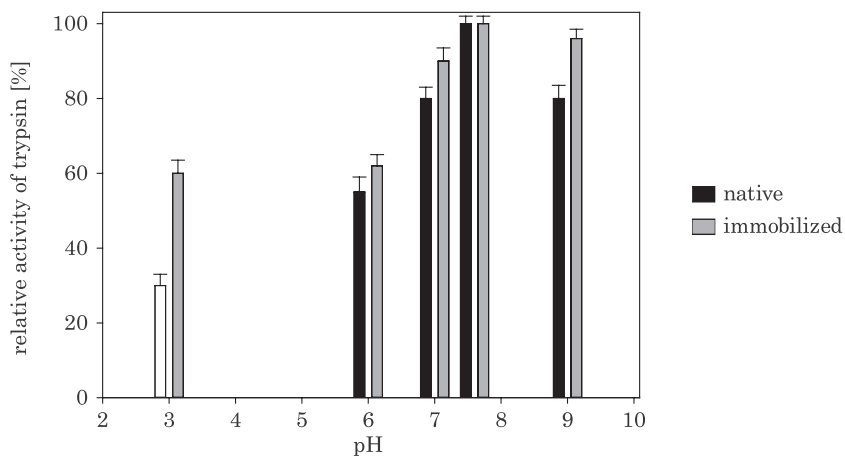

Fig. 3. The effect of pH on activity of native and immobilized trypsin

Moreover, the reusability of immobilized trypsin for hydrolysis of milk proteins was assayed. Reusability is one of the best advantages of enzyme immobilization. The reusability of immobilized trypsin was shown (Fig. 4).

Three different samples of immobilized trypsin were repeatedly used at least 8 times, and they all kept similarly high activity during such use. All of the results suggest that the immobilized trypsin displayed very good reusability.

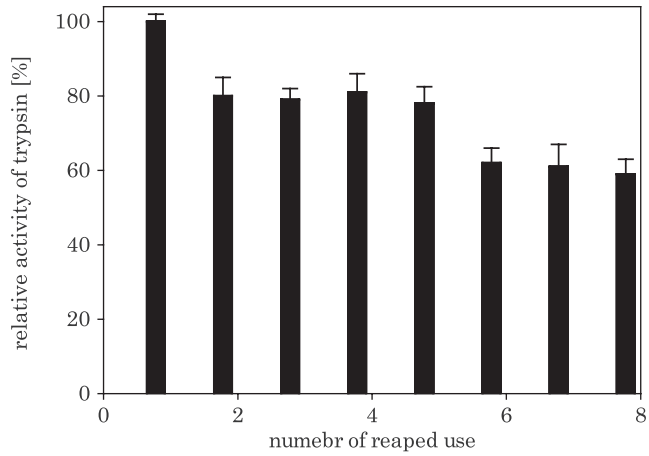

Fig. 4. Repeated uses of immobilized trypsin

During the test eight cycles were performed. In the cycles 2-5, activity of immobilized trypsin was 80%. In the cycles 6-8 it was equal to approximetly 60%.

The effect of storage time at 4℃ of immobilized trypsin on silica gel on activity was tested (Fig. 5). After 1, 3 and 14 days the activity decreased by approximetely 2%, 20% and 25% respectively.

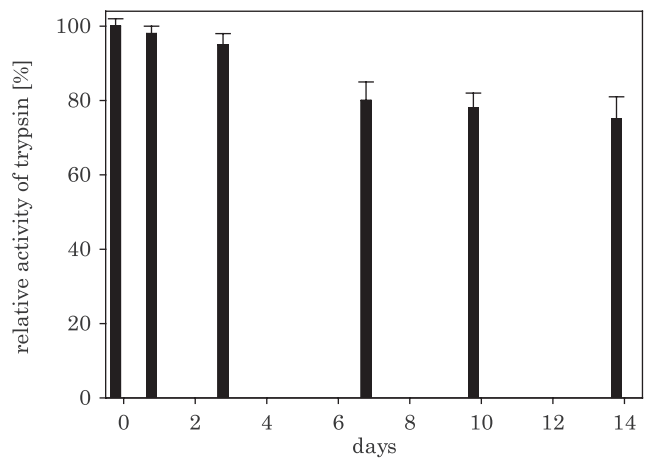

Fig. 5. The storage stability at the temperature of 4℃ on activity of immobilized trypsin

Conclusions

The aim of the study was to produce immobilized trypsin from bovine pancreas on modified silica gel in optimal conditions. Optimal conditions for production of immobilized trypsin on modified silica gel are as follows: activation time of 10% 3-aminopropyltriethoxysilane solution, 2.5% glutaraldehyde solution and trypsin solution is 120 minutes for each of the solutions. The trypsin immobilization on modified silica gel coating obtained higher thermal stability in temperature 65°C than native trypsin. Also, excellent reusability and long time storage in temperature of 4°C the immobilized trypsin illustrate the potential of this system of trypsin immobilized on silica gel for enzyme hydrolysis.

References

Endopeptidases: Advances in Research and Application: 2011. 2012. Ed. Q.A. Acton. A Scholarly Editions eBook, Atlanta, Georgia, p. 159-160.

Methods of Enzymatic Analysis. 2012. Volume 2. Ed. H.-U. Bergmeyer. Elsevier, Science, Academic Press, p. 1020.

Caramori S.S., de Faria F.N., Viana M.P., Fernandes K.F., Carvalho L.B. Jr. 2011. *Trypsin immobilization on discs of polyvinyl alcohol glutaraldehyde/polyaniline composite*. Materials Science and Engineering, C, 31: 252–257.

Gomez J., Romero M. 2009. *Adsorption of trypsin on commercial silica gel. Life* Sciences, 9(4): 336-341.

Mohamad N.R., Marzuki N.H.C., Buang N.A., Huyop F., Waha R.A. 2015. *Review. Agriculture and environmental biotechnology. An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes.* Biotechnology and Biotechnological Equipment, 29(2): 205-220.

Monteiro F.M.F., Silva G.M.M. 2007. *Immobilization of trypsin on polysaccharide film from Anacardium occidentale L. and its application as cutaneous dressing*. Process Biochemistry, 42: 884-888.

Salar S., Mehrnejad F., Sajedi R.H., Arough J.M. 2017. *Chitosan nanoparticles-trypsin interactions: Bio-physicochemical and molecular dynamics simulation studies.* International Journal of Biological Macromolecules, 103: 902–909.

Shen S.-C., Ng W.K., Chia L., Dong Y.-C., Tan R.B.H. 2011. *Sonochemical synthesis of (3-aminopropyl) triethoxysilane-modified monodispersed silica nanoparticles for protein immobilization*. Materials Research Bulletin, 46: 1665–1669.

Sun X., Cai X., Wang R.-Q., Xiao J. 2015. *Immobilized trypsin on hydrophobic cellulose decorated nanoparticles shows good stability and reusability for protein digestion*. Analytical Biochemistry, 477: 21–27.

Yang G., Wu J., Xu G., Yang L. 2010. *Comparative study of properties of immobilized lipase onto glutaraldehyde-activated amino-silica gel via different methods*. Colloids and Surfaces B: Biointerfaces, 78: 351–356.

Zhou C., Wu X., Jiang B., Shen S. 2011. *Immobilization strategy of accessible transmission for trypsin to catalyze synthesis of dipeptide in mesoporous suport*. Korean Journal of Chemical Engineering, 28(12): 2300-2305.

Zhao Q., Hou Y., Gong G.-H., Yu M.-A., Jiang L., Liao F. 2010. *Characterization of alcohol dehydrogenase from permeabilized brewer's yeast cells immobilized on the derived attapulgite nanofibers*. Applied Biochemistry and Biotechnology, 160(8): 2287–2299.