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Effects of pore characters of mesoporous resorcinol–formaldehyde carbon gels on enzyme immobilization

T. Chaijitrsakool [a], N. Tonanon [a], W. Tanthapanichakoon [a], H. Tamon [b], S. Prichanont [a,*]

[a] Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University, Bangkok 10330, Thailand
[b] Department of Chemical Engineering, Graduate School of Engineering, Kyoto University, Katsura, Kyoto 615-8510, Japan

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ABSTRACT

This paper demonstrates, for the first time, the use of resorcinol–formaldehyde carbon gels (RFCs) as enzyme carriers. The immobilization behavior of *Bacillus licheniformis* serine protease in RFCs of different pore characters was investigated. RFCs derived with (RF1) and without (RF2) cationic surfactant (trimethylstearylammonium chloride; C18) resulted in predominantly microporous, and mesoporous characters, respectively. It was found that support pore size and volume were key parameters in determining immobilized enzyme loading, specific activity, and stability. RF2, with higher mesopore volume (V_{mes}: RF1 = 0.21 cm^3/g; RF2 = 0.81 cm^3/g) and mesopore size radius (RF1 = 1.7–3.8 nm; RF2 = 7.01 nm), accommodated approximately fourfold more enzyme than RF1. Serine protease loading in RF2 could reach as high as 21.05 unit/g support. In addition, RF2 was found to be a better support in terms of serine protease operation and storage stability. Suitable mesopore size likely helped preventing immobilized enzyme from structural denaturation due to external forces and heat. However, immobilized enzyme in RF1 gave 12.8-fold higher specific activity than in RF2, and 2.1-fold higher than soluble enzyme. Enzyme leaching was found to be problematic in both supports, nonetheless, higher desorption was observed in RF2. Enhancement of interaction between serine protease and RFCs as well as pore size adjustment will be necessary for repeated use of the enzyme and further process development.

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1. Introduction

Mesoporous materials have recently demonstrated as attractive candidates for enzyme immobilization due to their fairly uniform, adjustable pore sizes and structures, large surface area and pore volume [1,2]. The ability to control pore size and characters of mesoporous materials is of immense advantage since these factors can exhibit direct impact on activity and stability of immobilized enzymes. For example, Kovalenko et al. [3] revealed that appropriate support pore size could result in effective and strong enzyme adsorption. Moreover, maximum stabilization of enzyme could be achieved by regulating mesopore size to a diameter of two to six times the diameter of immobilized enzyme [4]. So far mesoporous carbons have proven excellent matrices for enzyme immobilization since they possess optimum adsorption properties for enzymes in comparison to silica and metal oxide-based supports [5–7]. Their usages are, therefore, of wide applications such as biocatalysts, and biosensors [1,4].

Resorcinol–formaldehyde carbon gels (RFCs), a type of mesoporous carbon materials, could easily be synthesized and tailor-made for specific applications. Moreover, these materials (prepared by drying and pyrolysis of RF gel formed by polycondensation of resorcinol and formaldehyde) possess unique characteristics with potential application as enzyme carriers. Useful properties of RFCs are moderately high BET surface areas of 500–1200 m^2/g, and large mesopore volumes of greater than 0.89 cm^3/g [8]. Properties of RFCs such as particle and pore sizes, compactness of gel structure, surface area, and particle aggregation characteristic are governed by various parameters, for example, reactant concentrations, types and concentration of catalysts, and conditions of gelation, gel drying, and carbonization [9]. Although RFCs were previously applied as carriers for chemical catalyst immobilization [10], usage of these materials for enzyme immobilization has never been documented.

Since proteases are widely known as the most important industrial enzymes which account for roughly 60% of total enzyme market [11,12], *Bacillus licheniformis* serine protease (EC 3.4.21.62) was selected as a model enzyme in this work. The aim of this study was to investigate effects of RFC pore characters on enzyme loading, activity, and stability. Two types of RFCs with different

* Corresponding author. Tel.: +66 2 218 6860; fax: +66 2 218 6877.
E-mail address: seeroong.p@chula.ac.th (S. Prichanont).

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pore sizes, volumes, and structures were therefore synthesized and deliberately chosen as carriers for serine protease immobilization. Casein hydrolyses were followed for determination of both free and immobilized enzyme activities.

2. Experiment

2.1. Materials

Soluble serine protease from *B. licheniformis* serine protease (Novozyme EC 3.4.21.62) was kindly donated from East Asiatic Co. Ltd. Resorcinol ($C_6H_4(OH)_2$; 99%), and casein were from purchased from Fluka. Formaldehyde (HCHO; 36.5%), *t*-butanol (95%), and sodium carbonate (Na_2CO_3) were obtained from Ajax Finechem. Trimethylstearylammonium chloride ($CH_3(CH_2)_{17}N(CH_3)_3)Cl$) was procured from Tokyo Kasai. Trichloro acetic acid was purchased from Merck.

2.2. Preparation of resorcinol–formaldehyde carbon gels

Resorcinol–formaldehyde (RF) solutions were prepared from resorcinol (R), formaldehyde (F), sodium carbonate (C), and distilled water (W). The molar ratios of resorcinol to catalyst (R/C), and resorcinol to formaldehye (R/F), and the mass to volume ratio of resorcinol to water (R/W) were fixed at 400 mol/mol, 0.5 mol/mol, and 0.25 g/ml, respectively. The prepared RF solutions were first kept at gelation temperature (T_{gel}) of 333 K and the sol–gel transition continued. Before RF solutions lost their fluidity, they were dispersed into an aqueous solution of 0.4 mM cationic surfactant (trimethylstearylammonium chloride; [($CH_3(CH_2)_{17}N(CH_3)_3)Cl$]). After that, the mixtures were agitated at 850 rpm and 333 K for 2 h. RF hydrogels were then aged at 333 K for another 24 h before being kept at room temperature for 3 days. Solvent exchange was then carried out by immersing RF hydrogels in *t*-butanol for 3 days, and fresh solvent was replaced after each day of immersion. Next, RF hydrogels were microwave dried at 160 W for 25 min before being pyrolysed. Pyrolysis was conducted under a 200 cm^3-STP/min flow of nitrogen gas. Samples were heated up to 523 K with 250 K/h heating rate, and were kept at this temperature for 2 h. Heating was then continued at the rate of 250 K/h until 1023 K was reached and the temperature was kept constant for 4 h. RFCs designated as RF1 were finally obtained after the process.

RFCs synthesized without surfactant (designated as RF2) were prepared in the same way as above, however, aqueous surfactant solution was replaced by plain cyclohexane in the step to form inverse emulsion.

2.3. Characterization of resorcinol–formaldehyde carbon gel

The porous properties of RFCs were determined by nitrogen adsorption method using an adsorption apparatus (BEL Japan; BELSORP28). Adsorption and desorption isotherms were measured at 77 K, and the BET surface area (S_{BET}), mesopore volume (V_{mes}), and mesopore size distributions of RFCs were determined. The pore size distributions of the RFCs were obtained by applying the Dollimore–Heal method [13] to their desorption isotherms.

The surface and cross-section of RFCs were observed by a scanning electron microscope (JEOL, JSM6301F, Japan).

2.4. Immobilization of serine protease

In preparation for immobilization, RFCs were pre-wet with 50 ml ethanol for 30 min and pre-washed with 50% ethanol–water to exclude the air within the support particles. Next, the mixture was washed with 50 ml distilled water, decanted, and washed again with 100 ml fresh distilled water. Finally, RFCs were filtered and ready for enzyme immobilization.

Upon immobilization, 3 g of prepared RFCs was brought into contact with 30 ml serine protease solution (EC 3.4.21.62) (containing 10% (v/v) enzyme in 0.1 M carbonate–bicarbonate buffer solution; pH 10.5) in a sealed vessel. The vessel was then placed in an orbital shaker at 150 rpm and room temperature (30 ± 2 °C) for a certain length of time. Next, the enzyme-loaded RFCs were washed for three times with distilled water. They were then filtered, dried, and stored at 4 °C before further uses.

2.5. Assays of immobilized and free serine protease activities

The reaction was initiated by an addition of 1 ml of 1.0% (w/v) casein solution to a prepared mixture solution. The prepared solution contained 1.9 ml of 0.1 M carbonate–bicarbonate buffer solution (pH 10.5) and 3 g of enzyme-loaded RFCs. After reaction initiation, the reacting solution was incubated at 45 °C for 20 min. The reaction was then stopped by an addition of 2.0 ml of 10% (w/v) trichloroacetic acid solution and the reacting temperature was rapidly reduced using an ice-cold bath. After centrifugation at 3,500 rpm (Kubota, Kubota5100, Japan) for 20 min, the supernatant was analyzed by spectrophotometer (Spectronic instrument, 4001/4, USA) at 280 nm for serine protease contents. One unit of serine protease was defined as the quantity of enzyme which liberated the digestion product and gave an extinction coefficient at 280 nm equivalent to 1 μg of tyrosine per minute under assay conditions. Determination of protein content was achieved using Lowry's method. Reported results were average numbers of triplicate analyses.

For an assay of free enzyme activity, 0.1 ml of free enzyme solution (10% v/v alkaline protease) was used in place of 3 g of enzyme-loaded RFCs. All other procedures were carried out as described for the case of immobilized enzyme.

2.6. Stability tests

Operational stability was carried out by conducting five reaction cycles (6 h of reaction/cycle) using the same immobilized enzyme. After each cycle the biocatalyst was filtered and washed with 20 ml buffer solution (pH 10.5). The washed solution was then tested for detached enzyme activity, whereas the immobilized enzyme was again incubated with fresh substrate.

Storage stability for immobilized enzyme was tested at two temperatures which were at 4 °C and ambient temperature (30 ± 2 °C). Samples were taken every week for activity tests during the total storage time of 4 weeks.

3. Results and discussion

3.1. Characteristics of resorcinol–formaldehyde carbon gels

Two characters of RFCs synthesized with (RF1) and without (RF2) cationic surfactant (trimethylstearylammonium chloride; C18) were tested for serine protease immobilization in this study. Nitrogen adsorption and desorption isotherms of these two RFCs are shown in Fig. 1. It is evidently noticed that the two isotherms are markedly different. The results indicated that RF1 corresponded to IUPAC type I microporous material, while RF2 corresponded to IUPAC type IV mesoporous material. Dollimore–Heal method was applied to the isotherms for calculations of sample mesopore volumes. Results in Table 1 reveal that RF2 accommodated approximately fourfold higher mesopore volumes, and much larger pore size than RF1. However, BET surface areas were comparable

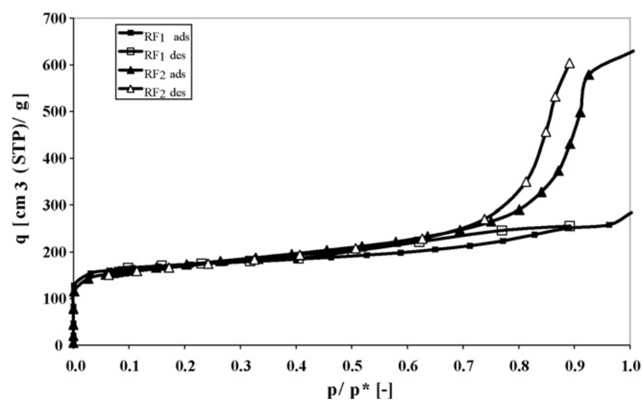

Fig. 1. Adsorption and desorption isotherms of nitrogen on carbon gels synthesized with and without trimethylstearylammonium chloride (RF1 and RF2, respectively) at 77 K; closed symbols, adsorption; open symbols, desorption.

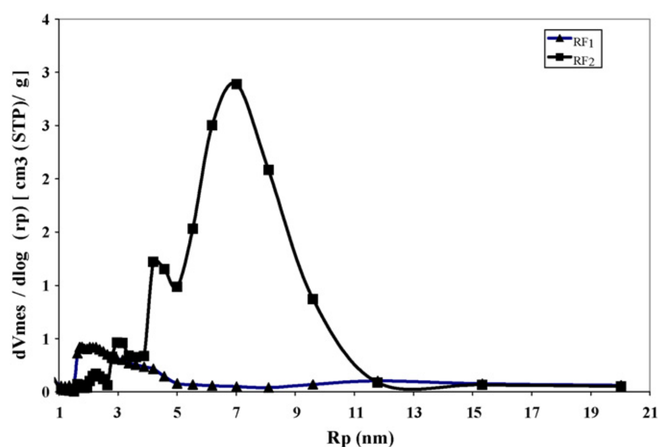

Fig. 2. Mesopore size distributions of carbon gels synthesized with and without trimethylstearylammonium chloride (RF1 and RF2, respectively).

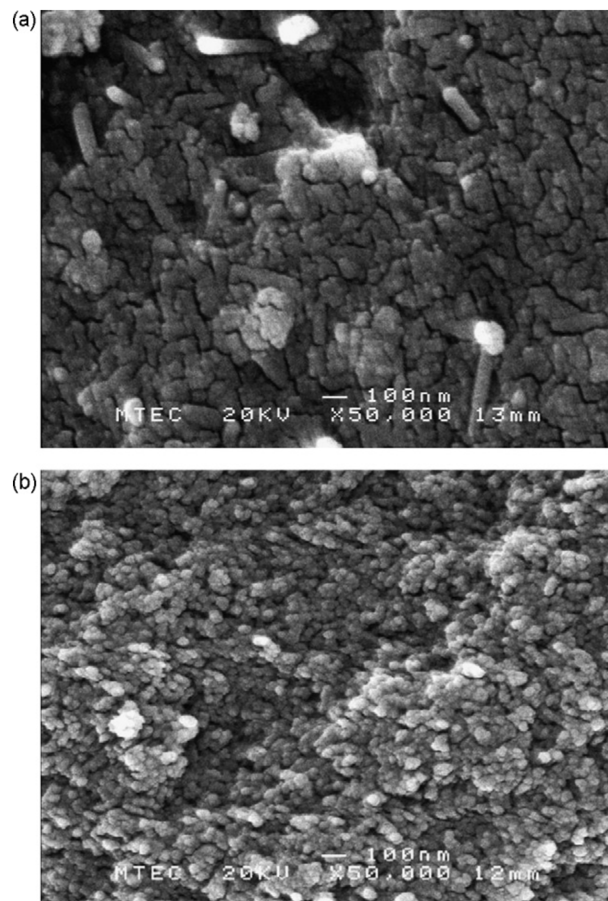

Fig. 3. SEM micrographs on the surfaces of carbon gels synthesized (a) with trymethylstearylammonium chloride (RF1) and (b) without trymethylstearylammonium chloride (RF2).

in both cases. Fig. 2 demonstrates wide mesopore size distribution of both RFCs. SEM micrographs of both RFCs disclose small, and fractal aggregates of both gel particles (see Fig. 3), but with more sphere-like aggregates in the case of RF2. For applications in enzyme immobilization, it would be interesting to compare RF1 and RF2 as enzyme carriers since they possessed different characters such as mesopore volumes, and mesopore sizes, but with similar BET surface areas.

3.2. Enzyme-loading capacity

Determination of enzyme loading was achieved by subtracting soluble enzyme activities at various time intervals from soluble enzyme activity at initial time. Fig. 4 demonstrates time courses of enzyme loading on RF1 and RF2. RF2 could load up to approximately 80% of initial enzyme amount while RF1 showed only one-fourth of the former's capacity. This is in accordance to the fourfold higher mesopore volume of RF2 than RF1. With an approximate diame-

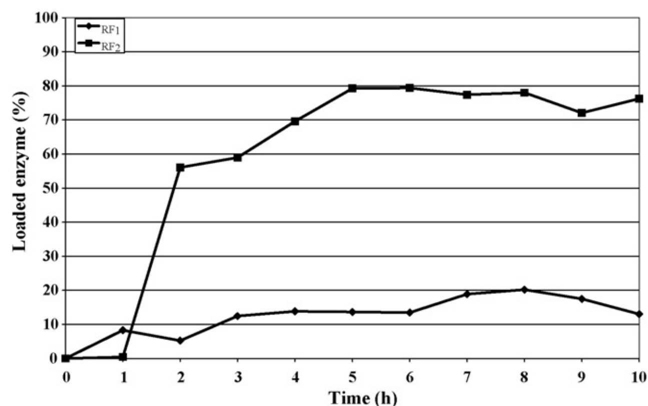

Fig. 4. Time courses of serine protease adsorption on carbon gels synthesized with and without trimethylstearylammonium chloride (RF1 and RF2, respectively). *Conditions*: 10% (v/v) initial soluble enzyme concentration, buffer solution pH 10.5, shaking speed 150 rpm, and room temperature (30 ± 2 °C).

Table 1
Synthesis conditions and porous properties of carbon gels

Sample	Trimethylstearylammonium chloride (mM)	S_{BET} (m^2/g)	V_{mes} (cm^3/g)	Mesopore size peak radius (mn)
RF1	0.4	518	0.21	1.7–3.8[a]
RF2	–	550	0.81	7.01

R/C = 400; R/W = 0.25 g/cm^3; R/F = 0.5; T_{gel} = 333 K; T_{pyro} = 1023 K.

[a] The mesopores with radii in the specified range accommodated 80% of the mesopore volume in RF1.

ter of 4 nm in all dimensions (measured from 3D structure of the enzyme), *B. licheniformis* serine protease trapping in micropores was certainly ruled out, and only mesopores of the supports could accommodate the enzyme. The size of serine protease was found to be comparable to those of RF1 mesopores (radius distribution of 1.7–3.8 nm). Therefore, higher mesopore volume and larger pore size of RF2 in comparison to RF1 (V_{mes} = 0.81 vs. 0.21 m^2/g; peak radius of 7.01 vs. distribution of 1.7–3.8 nm) resulted in higher enzyme loading. This is in accordance to previous publications which demonstrated that the amounts of loaded enzyme increase with increasing pore sizes [1,14]. Effects of different electrostatic interactions between serine protease and the two RFCs on loading capacity could be neglected. This is because the solvent exchange and pyrolysis at high temperature under inert atmosphere during RFCs syntheses probably resulted in electrostatic inertia of the two supports. Serine protease loading capacity of RF2 could reach as high as 21.05 unit/g support using 50% (v/v) enzyme solution in the immobilization process.

3.3. Activities of serine protease immobilized in RFCs

Specific activities of serine protease immobilized in RF1 and RF2 are compared with that of free enzyme in Fig. 5. It is obviously noticed that low enzyme-loading RF1 resulted in much higher specific activities (12.8-fold higher) than high enzyme-loading RF2. It was possible that the lower enzyme activity in RF2 was due to the steric impediments resulted from the high enzyme content. Moreover, it was likely to be caused the more matched sizes of RF1 pores and the enzyme than that of RF2. This is in accordance with Aburto et al. [15] who demonstrated that horseradish peroxidase showed higher activity when immobilized in mesopores with a pore size close to enzyme diameter. The results which show 2.1-fold higher activity of immobilized serine protease in RF1 in comparison to that of free enzyme is of no surprise. Similar observations have been reported for enzyme immobilization in mesoporous materials [16–18], and the explanation was that the favorable microenvironment of immobilized enzymes and the close contact with the immobilized surface could affect the enzyme superstructure and hence its activity. Therefore, 3D structure of serine protease was likely to be changed in favor of catalytic function when encaged in RF1 mesopores of relatively similar sizes (approximately one to two times larger than the enzyme size). Large mesopore sizes of RF2 with roughly 1.5–5.5 times (calculated based on distribution of mesopore radii) the enzyme size did not advantageously affect specific enzyme activity. On the other hand, high enzyme loading in the support could lead to steric impediments hence lower specific activity was observed.

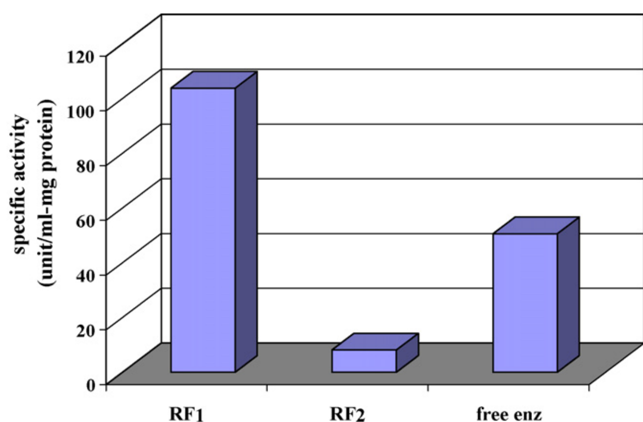

Fig. 5. Comparisons of specific serine protease activities between enzyme adsorbed on carbon gels synthesized with and without trimethylstearylammonium chloride (RF1 and RF2, respectively), and free enzyme. *Conditions*: 1.0% (w/v) initial casein concentration, buffer solution pH 10.5, and 45 °C.

3.4. Immobilized enzyme stability

Immobilized enzymes were tested for both operation and storage stability. Fig. 6 shows retention activity of immobilized and free serine proteases for each cycle of 6-h run. Although retention activity of immobilized enzyme in RF1 appears lower than that of free enzyme, activity losses were due mainly from enzyme desorption. Cumulative desorption of active enzymes from RF1 and RF2 after five reaction cycles were determined respectively at 21.2 and 43.4% of initially immobilized enzymes (data not shown). Therefore, activity losses due to enzyme denaturation after five reaction cycles could be verified at 68.7 and 18.4%, respectively, for RF1 and RF2 in comparison to 80.0% for free enzyme. This proves that RFCs helped preventing serine proteases from folding forces, thus operation stabilized them.

The fact that RF2 immobilized enzyme possessed much higher retention activity than that of RF1 was understandable. Theoretical calculations suggested that maximum stabilization of proteins could be obtained in spherical cages with a diameter of two to six times the diameter of native protein [4]. This was roughly the case of RF2 and serine protease (mesopore sizes 1.5–5.5 times the size of the enzyme). In contrast, RF1 mesopore sizes were only around one to two times the size of the enzyme, thus too small for maximum operation stability. A marked difference in activity losses due to enzyme denaturation for the case of RF1 (68.7% loss) and RF2 (18.4% loss) clearly suggested that RFCs should have pore diameters more than two times the diameter of serine protease for better stabilization of the enzyme. However, the optimum pore diameter for serine protease stabilization could not be determined from the results obtained in this study.

Although larger mesopore sizes of RF2 were of benefit in terms of operation stability, enzyme desorption from RF2 was significant in comparison to that of RF1. This was because serine protease was bound to RFCs only by weak van der Waals' forces, larger pore opening eased enzyme leaching.

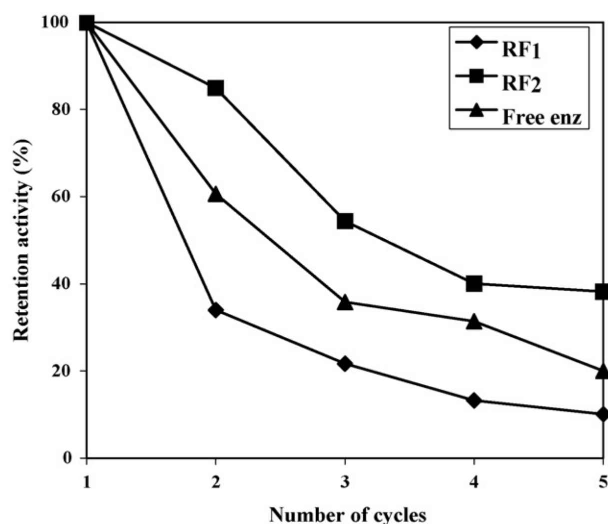

Fig. 6. Relation between retention activity of serine protease enzyme and cycle number of usages comparing between enzyme adsorbed on carbon gels synthesized with and without trimethylstearylammonium chloride (RF1 and RF2, respectively), and free enzyme.

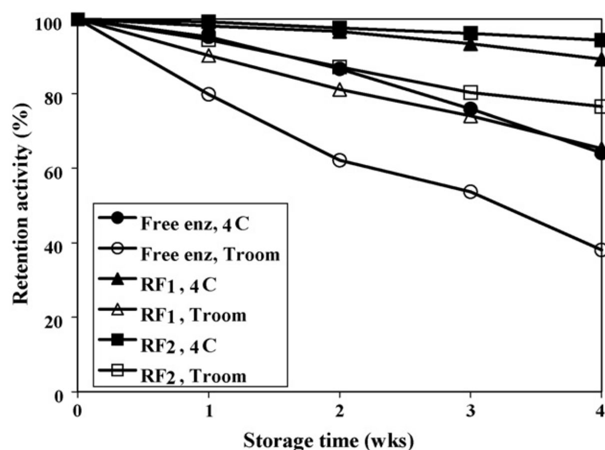

Fig. 7. Relation between retention activity of serine protease enzyme and storage time comparing between enzyme adsorbed on carbon gels synthesized with and without trimethylstearylammonium chloride (RF1 and RF2, respectively), and free enzyme at 4 °C, and room temperature (30 ± 2 °C).

Storage stability of both immobilized and free serine proteases was tested throughout a period of 4 weeks at 4 °C and ambient temperature (30 ± 2 °C). Fig. 7 shows that enzymes were better maintained at 4 °C than at ambient temperature in all cases. Activity of free enzyme was markedly reduced after 4 weeks of storage in comparison to those of immobilized enzymes. This was probably due to the prevention of thermal denaturation of enzymes by supports [19,20]. Similar to what was found in operation stability tests, RF2 was also found to be better enzyme carrier than RF1 in terms of storage stability. Immobilized serine protease in RF2 could retain as high as 94.3 and 76.6% of its initial activity after 4 weeks of storage at 4 °C, and room temperature, respectively.

4. Conclusions

Resorcinol–formaldehyde carbon gels (RFCs) of different pore characters were synthesized and used as supports for *B. licheniformis* serine protease immobilization. Mesopore sizes of RF1 and RF2 were respectively 1–2 and 1.5–5.5 times the size of the enzyme. In addition, RF2 mesopore volume was approximately four times that of RF1. It was found that support pore size and pore volume were key parameters determining immobilized enzyme loading, specific activity, and stability. Larger mesopore size and volume resulted in both higher enzyme loading and leaching after repeated uses. However, smaller mesopore size of RF1 gave better catalytic activity. Three dimensional structure of the enzyme was probably changed in favorable direction for catalysis, thus higher specific activity than soluble enzyme was achieved from enzyme-loaded RF1. In terms of operation and storage stability, RF2 was found a better support which was suggested to be due to their suitable pore size range in relation to the size of the enzyme.

This study demonstrated that optimization of support pore size is necessary for overall performance of the immobilized enzyme. In our case, the use of intermediate mesopore sizes of RFCs (sizes range in between those of RF1 and RF2) should lead to better serine protease overall performance (loading, specific activity, leaching, and stability). Surface modification of the support, however, will be necessary in order to improve enzyme loading while at the same time prevents enzyme leaching.

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