THE EFFECT OF STEM BROMELAIN IMMOBILIZATION BY COVALENT ATTACHMENT ONTO CNBr-SEPHAROSE 4B TOWARD ENZYMATIC ACTIVITIES AND STABILITIES

by:
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ABSTRACT:

The effect of stem bromelain immobilization by covalent attachment onto CNBr-Spharose 4B towards enzymatic activities and stabilities.

Rapid progress in biotechnology, bring about increased in need for enzymes, especially proteinase as biological agent, because of its broad range in functions, and therefore is applied in various industrial fields. Since the recovery yield and the reusability of free enzymes as industrial catalyst are quite limited, attentions has been paid to immobilized enzymes (IME), which offer advantages over free enzyme. Many approaches to the preparation of IME has been explrored and succesfully developed in recent years. Nevertheless, the covalent-immobilization method is known to be superior over the other methods, as far as stability is concerned. In attempt to modify the nature of enzymes, in this study Stem bromelain was attached covalently onto CNBr-Sepharose 4B, by the method modified from *Porath, et al*.

Key words: Immobilized enzymes, Stem bromelain, CNBr-Sepharose 4B

ABSTRAK

Pengaruh amobilisasi-kovalen bromelain batang pada *CNBr-Sepharose* 4B terhadap aktivitas dan stabilitas enzim.

Pesatnya kemajuan bioteknologi menyebabkan kebutuhan akan enzim, khususnya proteinase, sebagai agen biologi, semakin meningkat karena fungsinya

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yang sangat luas. Akan tetapi pemakaian enzim-bebas dalam industri tidak efisien karena stabilitasnya yang sangat terbatas. Amobilisasi-enzim sudah berhasil dikembangkan, namun metoda amobilisasi-kovalen lebih unggul di banding yang lain. Dengan teknik ini, enzim dapat digunakan berulang kali atau terus menerus, sehingga peranannya dalam bidang biomedik; analisis; industri dan riset akan lebih luas. Pada penelitian ini dilakukan amobilisasi-kovalen bromelain batang pada matriks pendukung CNBr-Sepharose 4B dengan modifikasi metoda Porath, dkk.

Kata-kata kunci: Amobilisasi-kovalen, Enzim-amobil, Bromelain batang

INTRODUCTION

Rapid progress in Biotechnology in the last few decades, bring about the increase in need for enzymes as biological agent to provide: products (goods) and services. The needs for proteinase in particular, will increase much more, because of its broad range in functions, and therefore is applied in various industrial fields.[1-3] However, since the recovery yield and the reusability of free enzymes as industrial catalyst are quite limited, attentions has been paid to immobilized enzymes (IME), which offer advantages over free enzyme. The advantages of IME may be summed up as follows as reasons for immobilizing enzymes: (a) Multiple or repetitive use of a single batches. (b) Ability to process on continuous basis. (c) Ability to stop reaction rapidly by removing the enzyme from the reaction solution. (d) Adaptibility to various engineering designs. (e) In many cases, the enzyme is stabilized by bonding. (f) The processed solution is not contaminated with the enzyme (immune responses, protein sequences and structure determinations. (g) Analytical purposes : long half-life, predictable decay rate, elimination of reagent preparation. In addition, enzymes on a miligram basis of pure enzyme protein, are perhaps the most expensive and difficult materials to obtain in reasonable quantities. Therefore, any procedure that can economically extend the life time of these biologically active molecules should be considered.[4-5] Many approaches to the preparation of IME has been explrored and succesfully developed in recent years.[6-8] Nevertheless, the covalent-immobilization method is known to be superior over the other methods, as far as stability is concerned. With this technique, the enzymes were not only made suitable for repeated use, but also can be made suitable for continuos used, thus making their role in the fields of : biomedical, analysis, industry and research more extensive.[9-12] In practice: Enzyme Immobilization is a method to modify a water-soluble (mobile) enzyme into water-insoluble (immobile) form, to make it more suitable for repeated use.[13] In attempt to modify the nature of these enzymes, in this study Stem Bromelain was attached covalently onto CNBr-Sepharose 4B, by the method modified from *Porath, et al.* [14]

MATERIAL AND METHOD

The main materials in this study were: Stem bromelain (EC:3.4.22.4) and CNBr-Sepharose 4B, as enzyme and matrix materials (carrier) respectively. Casein, as high molecular weight subtrate and BA*p*NA (Benzoil Arginin *para* Nitro Anilide), as low molecular weight substrate. All of those materials in pro-analytical quality and were purchased from Sigma Chemical Co, USA

The immobilization procedure was performed through a coupling reaction as follows: One gram CNBr-Sepharose 4B that was already washed and swollen, was suspended in 5 mL of 0.1 mM buffer solution of NaHCO3 at pH 8.0 that contains 0.5 M NaCl, 5mM mercaptoetanol and 2 mM EDTA. To the suspension, ten miligrams of enzyme protein (stem bromelain) was added under stirring and the mixture was kept at 4EC for 16 hours under rotation. The product of coupling reaction was carried out through a washing process by repeated centrifugation. Finally, after an overnight freeze-drying process, a matrix-enzyme conjugate was obtained in the form of dry powder. The whole immobilization process was repeated three times.

The immobilization process was evaluated with respect to: The yield (total recovery) and protein concentration of IME. Protein measurement was carried out according to the method of : Lowry *et al.*[15] Proteolytic activity (toward casein) and esterolytic activity (toward Ba*p*NA), were determined according to the method of *Bergmeyer* and *Erlanger, et al* respectively, with minor modification to overcome some special problems encountered with the insoluble conjugates.[16,17] The stability were tested towards three kinds of effect such as reuse, heat and storage stability. Reuse stability was determined in accordance with the method of *Hayashi* and *Ikada.*[18] The dried IME were washed twice in 0.1 M PBS, and then suspended again in a fresh reaction mixture to measure the enzymatic activity. This cycle was repeated on the same sample. To check the possibility of any leakage of enzyme under washing, the amount of IME was determined after the last batch test.

The heat stability was determined according to the method of of *Ulbrich* and *Schellenberg,*[19] by measuring the residual activity of enzymes exposed to various temperature in 0.1 M PBS of pH 7.2 for 1 hour. After cooling, the solution or suspension of enzyme was assayed for its enzymatic activity. The storage stability of free enzyme and IME in buffer was evaluated by placing the enzymes in 0.1 M PBS of pH 7.2 at 32EC and 4EC for periods of time and the activity was assayed using above mentioned techniques.

RESULT AND DISCUSSION

The average total yield of 426.5 mg powder of IME was obtained, which cointains 5.12 mg (51.2%) enzyme proteins (Tabel: 1). This result showed a substansial decrease

of matrix materials has occurred. The repeated-centrifugation during gel washing process was considered to be the main cause, so some part of gel broke or became damaged. Centrifugation may also cause some enzymes detached from the matrix and then cause a relative low yield of enzyme proteins. However in general, the total yield (enzyme-matrix conjugates) as well as the enzyme-proteins is still comporable with what has been reported by other researchers.[20,21]

Tabel: 1 The result of Immobilization of Stem Bromelain (EC. 3.4.22.4) by covalent attachment onto CNBr-Sepharose 4B.

N	Yield (mg)	Protein Concentration (mg/ml)	Total Protein (mg)	Enzyme Protein (%)
1	391.2	0.125	4.89	48.9
2	421.8	0.120	5.06	50.6
3	466.4	0.116	5.41	54.1
($\bar{x}$)	426.5	0.120	5.12	51.2

Condition were: Coupling reaction between : 1 g CNBr-Sepharose 4B with 10 mg enzyme protein in buffer solution of 0.1 M NaHCO3; pH 8.0 that contains 0.5 M NaCl; 5 mM mercaptoethanol and 2 mM EDTA, for 16 hours at 4EC.

From the activity test, a relative activity toward casein and BA*p*NA were 34.7% and 62.4%, respectively (Tabel: 2,3). The proteolytic activity was considered sufficient or relatively high, since in general, the activity of IME toward high molecularweight substrate was indeed low. On the contrary, activity toward Ba*p*NA was somewhat low, presumably due to less sensitively of Ba*p*NA assay or less actively of IME in breaking amide compound. The difference of activity towards two kinds of this substrates, indicated the existence of steric hindrance and diffusion limitation in the assay of IME toward high molecular weight sub strate.[22]

Durability of IME is very important in application, because they are subject to be repeated in hydrolisis reaction. At the end of reuse test (N=10), the residual activity is seen to be retained at 79.3%, decreased about 20% compare with original one. During the test, a non-linear decline in activity was observed (Fig. 1). The half life of IME can not predicted, unless continuation of the test until activity drop to 50 %.

Tabel: 2 The activity of immobilized stem bromelain toward casein as substrate

N	Protein Concentration (mg/mL)	Total activity (Unit/mL)	Spec.activity (Unit/mg)	Relative activity (%)
1	0.125	4.71	37.68	31.32
2	0.120	4.53	37.75	34.24
3	0.116	4.89	42.16	38.57
($\bar{x}$)	0.120	4.71	39.23	34.70

Condition : Absorbance were observed at 275 nm.

Tabel: 3 The activity of immobilized stem bromelain toward BA*p*NA as substrate

N	Protein Concentration (mg/mL)	Total activity (Unit/mL)	Spec.activity (Unit/mg)	Relative activity (%)
1	0.125	0.53	4.24	58.24
2	0.120	0.52	4.33	61.16
3	0.116	0.55	4.74	67.91
($\bar{x}$)	0.120	0.53	4.44	62.40

Condition : Absorbance were observed at 410 nm.

This result was still within the acceptable limit.[23] The thermal stability of IME is one of the most important criteria of their application. As is well known, the activity of IME preparations especially in covalently bound systems, is more resistant against heat and denaturing agents than that of free enzyme. The effects of temperature on stability of IME in solution are shown in Fig.2. At any temperature IME showed more resistant against heat than that of free enzyme. The residual acitvity of IME at 60EC was 89%, whilst in the case of free enzyme was about 64%. At 70EC both forms of enzymes showed a severe decreased in activity, but the IME still have activity of about 40%, whereas of free enzyme remains 18%. This result indicated IME to be unstable at a temperature higher than 60EC. .

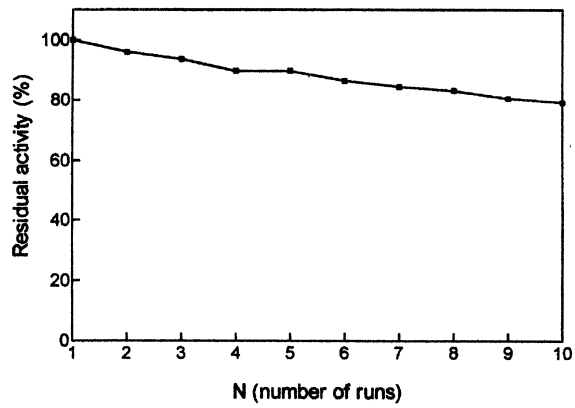

Figure 1. Effect of repetead use on residual activity of casein hydrolysis at pH 72

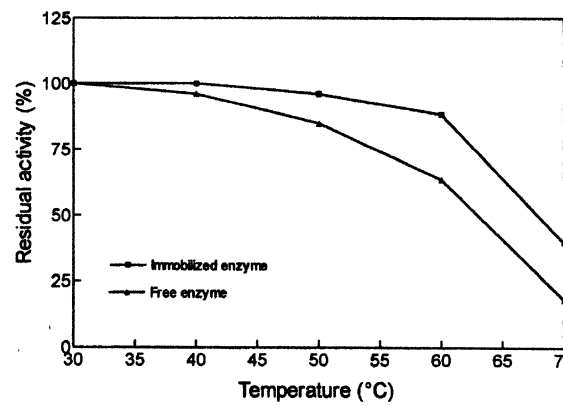

Figure 2. Effect of the heat teatment at given temperature for 1 hour 0n the residual activity of casein hydrolysis

Similar result were obtained towards the effects of storage. The IME showed a higher stability at a room temperature as well as at low temperature. After storage for 6 weeks at 32EC, the residual activity of IME became 46.1 %, whereas at a low temperature the activity became 80.8 %. The corresponding residual activities of free enzymes were 9.1% and 44.8 % respectively (Tabel: 4). The higher stability of IME can be attributed in the prevention of autodigestion and thermal denatutarion as a result of fixation of stem bromelain on the suface matrices.[18]

Tabel: 4 Effects of storage in 0.1 M PBS , pH 7.2 at 32EC and 4EC on the residual activity of hydrolysis at pH 7.2

Time (Week)	Residual activity (%)			
	Immobilized enzyme		Free enzyme	
	32°C	4°C	32°C	4°C
0	100	100	100	100
1	96.2	100	82.5	92.4
2	86.7	96.2	56.6	76.9
3	78.9	92.3	37.7	69.2
4	65.3	88.5	23.8	58.7
5	55.9	84.7	17.5	52.4
6	46.1	80.8	9.1	44.8

CONCLUSIONS

From the result obtained above, some conclusions can be taken as follows: In principle, the covalent immobilization process of Stem Bromelain onto CNBr-Sepharose 4B has been succesfully done, resulting in a relatively low yield of enzyme-matrix conjugates and enzyme proteins. Presumably due to inability of the matrix materials to endure the mechanical influence of the repeated centrifugation.

1. Even though it was not quite satisfying, the proteolytic activity of IME was sufficiently high, and the esterolytic activity was relatively low. The low of esterolytic activity may be due to the fact that the enzyme has less affinity toward Bap*p*NA.
2. Although the IME showed the existence of steric hindrance and diffusion limitation, in general the immobilization process did not cause drastic changes to the conformational of the enzymes molecules, so in other words the condition of the coupling reaction were suitable.
3. Durability for repeated use of the IME were sufficiently good, however a small part of enzyme molecules were lost from its matrix bond, due to repeated washing by centrifugation.
4. The heat and storage stability of the IME were higher than that of free enzymes.The higher stability may be attributed to the prevention of autolysis and thermal denaturation as a result of fixation of enzyme molecules on the matrix surface.

SUGGESTIONS

1. Test of stability need to be continued, in particular the stability at continuous use. In addition, the stabilty towards various inhibitors, denaturing agents and proteolytic enzymes, need also to be tested.
2. The study to find out the cause of low yield of enzyme-matrix conjugates and the enzyme-proteins, should be carried out.
3. The study to increase the activity of IME, by : Addition of inhibitor to the coupling reaction; use of spacer molecules of binding through the carbohydrate moeity of the enzyme molecule.

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