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Production of Aspartame by Immobilized Thermolysin

Mohammed A. Alsoufi [1*], Raghad A. Aziz [2]
[1]Department of Evaluating Products, Market Research and Consumer Protection Centre, University of Baghdad, Baghdad, Iraq
[2]Department of Science, College of Basic Education, University of Al-Mustansiriah, Baghdad, Iraq

Abstract

The aim of this study was the production of aspartame by using immobilized thermolysin in bentonite clay. The yield of immobilized thermolysin in bentonite was 92% of the original enzyme amount. pH profile of free and immobilized enzyme was 7.0 and 7.5 respectively which was stable at 6.5-9.0 for 30min. The optimum temperature of both enzymes was 50°C, while they were stable at 65°C for 30min. however, they lost 52.73 and 61.72% from its main activity at 80°C respectively. Immobilized thermolysin has retained all activity within 27 days, but it kept 68.27% of initial activity when stored for 60 days at 4°C whereas, it retained a full activity after 20 continue usage. In addition, it retained 86.53% of its original activity after 30 continuing usages. The yield of produced aspartame was increased with reaction time; it was 9% after 1h and increased gradually to 100% after 10h at reaction conditions.

Keywords*: Immobilized enzymes, Thermolysin, Bentonite, Aspartame precursor, Enzymes applications.*

إنتاج سكر الأسبارتام باستعمال أنزيم Thermolysin المقيد

محمد عبد الرزاق الصوفي[1]*، رغد أكرم عزيز[2]
[1]قسم تقويم السلع، مركز بحوث السوق وحماية المستهلك، جامعة بغداد، بغداد، العراق
[2]قسم العلوم، كلية التربية الأساسية، الجامعة المستنصرية، بغداد، العراق

الخلاصة

هدفت الدراسة الحالية الى إنتاج مادة الاسبارتام باستعمال أنزيم theermolysin المقيد بطين، إذ احتفظ الأنزيم المقيد بـ92% من كميه الأنزيم المستعمل. بلغ الرقم الهيدروجيني الأمثل لفعالية الأنزيم الحر والمرتبط 7.0 و7.5 على التوالي، بينما اظهر كلاهما ثباتا تراوح بين 6.5–9.0 لمدة 30 دقيقة. بلغت درجة الحرارة المثلى لفعالية الأنزيم الحر والمرتبط 50م، واظهر كلاهما ثباتا كاملا عند 65م لمدة 30 دقيقة، لكنهما فقدا 52.73 و61.72% من فعاليتهما الأصلية بدرجة حرارة 80م على التوالي. احتفظ الأنزيم المقيد بكامل فعاليته بعد 27 يوم بينما احتفظ بـ68.27% من فعاليته الأصلية بعد فترة خزن 60 بلغت يوما عند درجة حرارة 4م. كذلك لوحظ أن الأنزيم المقيد احتفظ بكامل فعاليته بعد استعماله 20 مستمرة، بينما بلغت فعاليته 86.53% من الفعالية الأصلية بعد استعماله 30 مستمرة. أظهرت نتائج تطبيق الأنزيم المقيد أن حصيلة إنتاج الاسبارتام ازدادت تدريجيا بزيادة زمن التفاعل، إذ بلغت 9% من الحصيلة الكلية بعد مرور ساعة واحدة لتصل إلى 100% بعد مرور 10 ساعات من التفاعل في ظروف التجربة.

*Email: alsoufim@mracpc.uobaghdad.edu.iq

Introduction

Thermolysin (EC 3.4.24.27) is considered a thermostable and zinc-dependent neutral metalloproteinase, which is present in bacterial sources [1], and work as a catalyst for hydrolysis or synthesis of peptide bonds [2], therefore, it used widely to bioactive peptides produce during formation of bond or hydrolysis of protein [3], thermolysin has a high specific action against of amino acids which contain bulky and hydrophobic side chains [4], so, the most using of thermolysine include peptide preparation of N-carbobenzoxy L-Asp-L-Phe methyl ester (ZDFM) by coupling, N-carbobenzoxy L-Asp (ZD) and L-Phe methyl ester (PM) to formation a precursor which consider as a starter to artificial sweetener production [5]. Aspartame its combining of aspartic acid and phenylalanine which discovered in 1969 as sweetener that has "sweetening up to 200 times more than to table sugar with 4 kcal/g which use in many consumer products [6], such as tabletop sweeteners, yogurt, chewable multi vitamins, soft drinks, breakfast cereals and pharmaceuticals [7], due to its good sensory properties, such as, safety to high temperature, non carcinogenic and acceptable from consumer [8].

Immobilized enzymes were used in many industrial applications, such as, foods, pharmaceuticals, detergents and textile [9], this technique provides several advantages for enzymes, such as, improving catalytic properties, reducing of production cost, possibility of using them more than once and the promote and increase stability [10, 11]. Several methods have been reports to immobilization of thermolysin, such as, covalent, adsorption and other methods [5], covalent linkage of enzymes with immobilizing material happen to "side chain amino acids, such as, aspartic acid, arginine, and histidine, the degree of reactivity depended on functional groups, such as, phenolic hydroxyl, imidazole, indolyl, etc. [12, 13].

Bentonite clay (Montmorillonite) is known as the most popular clay rocks in the world that widely used as a support in different applications, the nature of this clay was acidic which provide acid sites for adsorption of enzymes through NH_2 group [9]; the clay also, can be activated with glutaraldehyde to make covalent bond with enzyme [13]. Due to republic of Iraq possession a huge quantity of this clay in the Western regions, so, the aim of this study was to use bentonite as a substance to immobilize thermolysin and study some effects of its activity and its application for aspartame precursor production.

Materials and methods

Chemicals

Lyophilized powder of thermolysin from *Geobacillus stearothermophilus* (Sigma-Aldrich), Bentonite, was obtained from local market in Baghdad.

Thermolysin assay

Azocasein was used as a substrate for free and immobilized thermolysin activity. 4mL of (0.5%) substrate solution was mixed with enzyme solution (2g/L of thermolysin in 50mM phosphate buffer at pH 7.0), and the reaction was continued until proteolysis of the casein releasing a soluble dye into solution. The reaction was stopped by adding 500µL of 15% trichloroacetic acid to a 1mL aliquot of the reaction solution, then centrifugation at 10000rpm for 15min. The precipitate was removed and enzymatic activity was measured at 440nm. One unit of enzymatic activity was defined as an amount of thermolysin producing an increase of 0.1 absorbance units/min at 440nm [2, 3]

$$\text{Units/ml enzyme} = \frac{\Delta A440nm\ \text{Sample} \times T.V}{T \times V}$$

∆A440nm Sample = A440nm Test – A440nm Sample Blank

TV = Total volume (in milliliters) of assay

T = Time of assay (in minutes) as per the Unit Definition

V = volume of enzyme (in milliliter) of enzyme used

Protein estimation

Protein (mg/ml) was estimated through a method of Bradford [14].

Activation of bentonite clay

Bentonite was activated by stirring with 10% of 3-APTES solution in acetone (v/v) for 1h at room temperature. The mixture was then filtered, washed with acetone and dried at 80°C. Then the mixture was treated with 10% aqueous glutaraldehyde solution (v/v) for 1h and filtered, washed, dried at 25°C and stored in 20mM phosphate buffer pH 7.0 at 4°C until use [13].

Immobilization of thermolysin:

Twenty mL of enzyme solution (2g/L thermolysin in 50mM phosphate buffer at pH 7.0) was mixed with 500mg of bentonite clay and stirred at 4°C for 12h; then centrifuged at 10000rpm for 10min. The supernatant (free enzyme) was removal and precipitate (immobilize enzyme) was washed three times with 20mL of 100mM phosphate buffer pH 7.0; centrifuge was used after each wash to ensure removal all free enzyme. The immobilized enzyme was stored in 20mM phosphate buffer pH 7.0 at 4°C until use [2].

Immobilization yield

Immobilization yield was estimated by measuring the ratios of free thermolysine solution which added to bentonite (At_0) and the ratio of immobilized enzyme in bentonite after stirring the mixture at 4°C for 24h (Att). The immobilization yield (IY) was calculated with the following equation [11].

$$IY\ (\%) = \frac{At_0 - Att}{At_0} \times 100$$

Characterization of enzyme

Effect of pH on free and immobilized thermolysin activity

The effect of pH on the activity of the free and immobilized thermolysin was determined by preparing substrate (buffer containing 0.5% azocasein) in different buffer solutions include 50mM sodium acetate buffer, 50mM sodium phosphate buffer, 50mM Tris/HCl buffer and 50mM Tris-base buffer to obtain (pH 5,5.5,6,6.5,7,7.5,8,8.5 and 9). Free and immobilized thermolysin activity was determined at 37°C by using 0.5ml and 10mg and the activity was measured according to the method described by [15].

Effect of pH on free and immobilized thermolysin stability

An equal amount (v/v) and (w/v) from free and immobilized enzyme were mixed with the buffers at different pH (5-9) at a ratio of (1:1) and the mixture was incubated in a water bath for 30 min at 37°C. Then the samples were transferred directly to the ice bath. The remaining activity (%) was estimated by Al-Soufi (9).

Optimum temperature on free and immobilized thermolysin activity

Free and immobilized thermolysin activity was estimated at a different range of temperature ranging from 30 to 80°C using 50mM Tris/HCl buffer solution in pH 7.5 for 10min [15].

Effect of temperature on free and immobilized enzyme stability

Immobilized thermolysin was incubated at different temperatures (30-80)°C for 30min. Followed by incubation in ice bath. Remaining activity (%) of immobilized thermolysin was estimated by Al-Soufi [9].

Synthetic aspartame precursor

Immobilized thermolysine was equilibrated with 0.05M MES-NaOH buffer containing 5mM $CaCl_2$ in pH 7.5 to synthetic aspartame precursor. 2g/100mL of immobilized enzyme was added to 10mLof solvents mixture [tert-amyl alcohol (TA) and ethyl acetate (EA)] at various ratios, containing 40mM of Z-Asp and 200mM of PheOMe as the substrate. The reaction was left for 10h at 40°C and the absorbance was estimated at 254nm [16, 17], the yield of aspartame precursor forming [N-(B enzyloxycarbony1)-L-aspartyl-L-phenylalaninme ethyl ester (Z-AspPheOMe)] (%) was calculated as the following equation:

$$Yield\ (\%) = \frac{A}{B} \times 100$$

A = absorbance at 254nm of reaction at hour.

B = absorbance at 254nm of reaction at 10 hour.

Results and discussion

Yield of immobilization

The yield of immobilized thermolysin with bentonite was 92% from the original enzyme amount, which was largely encouraging to be used in this work. This result is similar to what reported by Moeschel *et al.* [18] who referred that all type of spacers and activation reagents gave high yields of immobilized thermolysin on polyamide nonwoven materials. However, in contrast, Mateo *et al.* [19] found that recovered activities and stabilization factors was 100% for immobilized thermolysin on glyoxyl agarose. Chen *et al.* [5] reported that 93.2% of thermolysin was linked to the sodium chloride

which uses as a immobilize material, while, it was more than David *et al.* [2] who referred that maximum loading of Immobilization was 733±13 mg thermolysin per 1000mg of glutaraldehyde-activated silica gel GA-N-CSMG. Therefore, it can be said that the high loading capacity of the bentonite will allow linking high amount of enzyme units, which lead to use of minimum immobilized thermolysin for production of aspartame.

Characterization of enzyme

Optimum pH of activity and stability of free and immobilized thermolysin

The pH profile of free and immobilized thermolysin activity was 7.0 and 7.5 respectively (Figure-1), and both enzymes were stable at pH range 6.5-9.0 for 30 min (Figure-2).

Optimum pH profile of enzymes was considered as an important parameter of immobilization. The variation of pH value may cause inability to use immobilized enzyme in high efficiency, so, the feasibility of immobilization will be ended, and the use of free enzyme will be best [11, 13].

Many authors have reported this effect, such as Yun [20] who referred that the optimum pH for free and immobilize thermolysin on Dowex MWA-1 with 10% glutaraldehyde and incorporated into a fluidized bed was 8.0 and 7.0 respectively, and the immobilized enzyme was stable more than free enzyme at alkaline ph. Similarly, Moeschel *et al.* [18] noted that immobilized thermolysin by polyamide nonwoven materials showed a remarkable improved stability with respect to elevated extreme pH values. On the other hand, David [15] explained that pH activity profiles for free and immobilized thermolysin on nanoporous silica gel were showed maximum activity at pH 7.0, while David *et al.* [2] found that maximum pH activity of free and Immobilization thermolysin with glutaraldehyde-activated silica gel was 7.5.

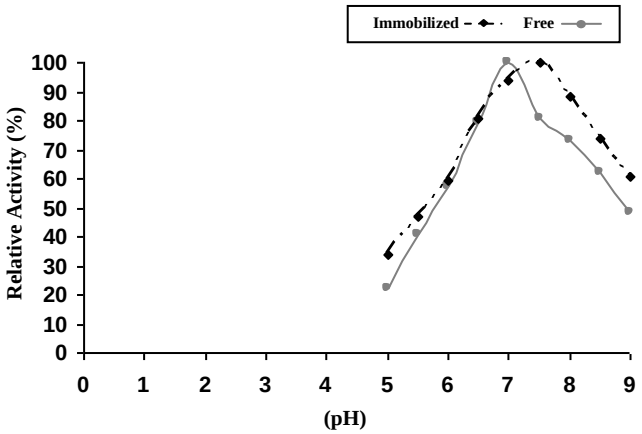

Figure 1- Effect of pH on activity of free and immobilized thermolysin on bentonite

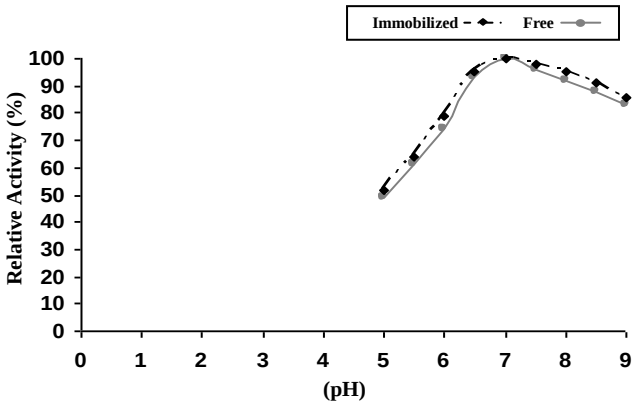

Figure 2- Effect of pH on stability of free and immobilized thermolysin on bentonite.

Optimum temperature of activity and stability of free and immobilized thermolysin

Optimum temperature for free and immobilized thermolysin activity was 50°C (Figure-3). Both enzymes were stable at 65°C for 30 min, but they lost 61.72 and 52.73% respectively, from its initial activity at 80°C at the same time (Figure-4).

High temperature causes a decrease in enzyme activity as a result of thermal denaturing due to the effect on open folds of the enzyme molecule and exposure of content from amino acids to the reaction medium [11]. Though, most immobilization studies were referring to a significant improve thermal stability for immobilized thermolysin with different materials [18, 5], On this basis, Yun [20] found that the optimum temperature of free and immobilize thermolysin on Dowex MWA-1 with 10% glutaraldehyde and incorporated into a fluidized bed was 70 and 80°C respectively. The immobilized enzyme was stable more than free enzyme at high temperature, while, David *et al.* [2] referred that the free enzyme showed an increase in its activity up to 65°C. Whereas, the immobilized thermolysin with glutaraldehyde-activated silica gel showed a huge increase in its activity up to 85°C

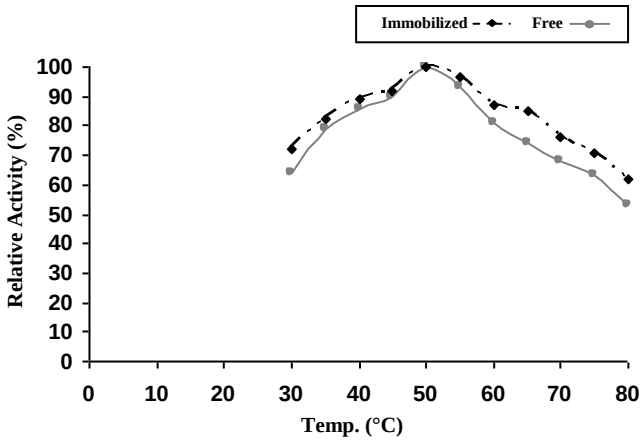

Figure 3- Effect of temperature on activity of free and immobilized thermolysin on bentonite.

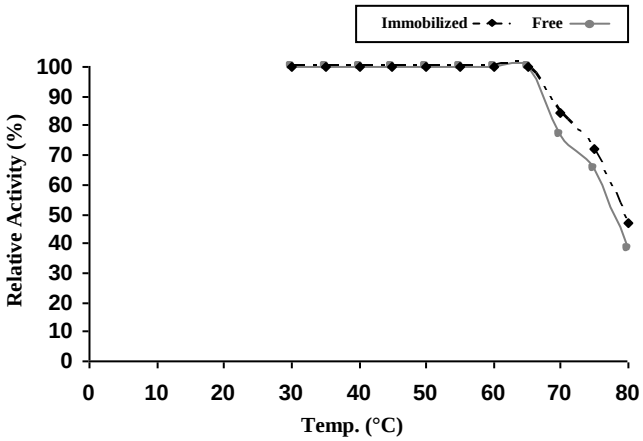

Figure 4- Effect of temperature on stability of free and immobilized thermolysin on bentonite.

Storage and Reuse of immobilized thermolysin

Immobilized thermolysin kept its activity for 27days, but it kept 68.27% from initial activity after storage for 60 days at 4°C (Figure-5). It retained a full activity for 20 continue usage; while it retained 86.53% of its original activity after 30 continue usage (Figure-6).

The Storage stability and reuse of immobilized enzymes represent one of the main economic factors in the immobilization because it gives a clear conception about the efficiency of materials which are used for this purpose [11].

In this context, Persichetti *et al.* [21] observed that crystals of thermolysin which immobilized by cross-linked method was stable in terms of activity for several hundred hours with a very low enzyme consumption rate. The soluble thermolysin lost 50% of its activity when it stored in a mixed aqueous organic solution within the first day of incubation, but then it remains relatively stable for the next 15day. Likewise, Belyaeva *et al.* [22] found that the immobilized thermolysin on polyvinyl alcohol cryogel was retained about 95% of original activity after 4 months of storage in 0.05M Tris-HCl buffer that containing 50mM $CaCl_2$, pH 7.2 at 4°C. Whereas, Dridi *et al.* [23, 24] referred that

immobilize thermolysin by cross linking glutaraldehyde onto gold interdigitated microelectrodes was high stable during 30 days of storage at 4°C in 20 mM phosphate buffer pH 7.0. Furthermore, Nagayasu *et al.* [16] noticed that the immobilized enzyme on Amberlite XAD-7 was stable for over 300h of usage at 45°C without any loss of activity.

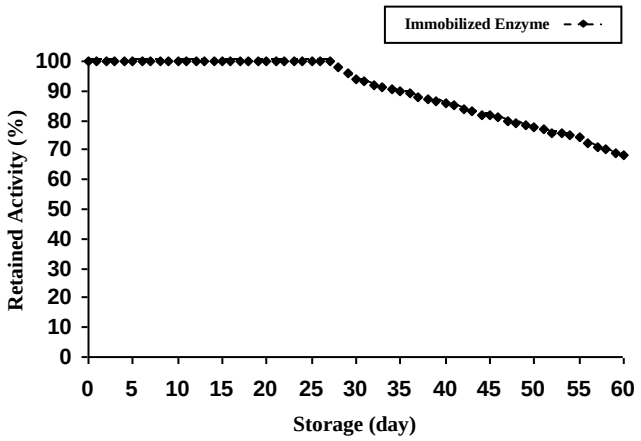

Figure 5- Effect of different period of storage at 4°C of immobilized thermolysin on bentonite.

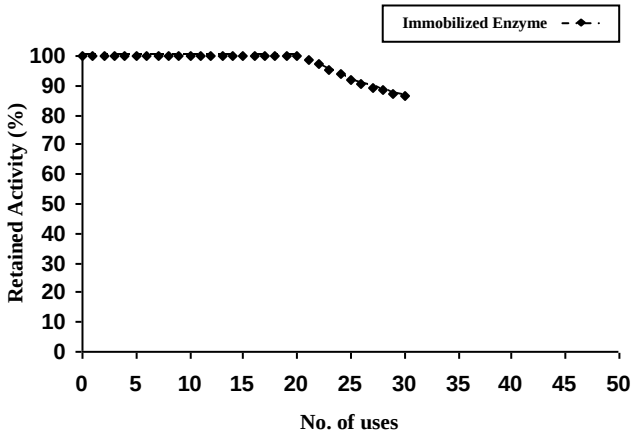

Figure 6- Effect of reuse of immobilized thermolysin on bentonite.

Application

The yield of aspartame precursor N-(B enzyloxycarbony1)-L-aspartyl-L-phenylalaninme ethyl ester (Z-AspPheOMe) was increased at the time of reaction (Figure-7). It was 9% after 1h and then increased gradually to be 100% after 10h at reaction conditions.

Immobilized thermolysin was used for the production of aspartame precursor and it has others applications. The enzymatic synthesis method was preferred for commercial production of aspartame compared with chemical syntheses method because the reaction can be increased with high specificity and consequently give a high yield. In this context, Nagayasu *et al.* [16] prepared aspartame precursor by using immobilized thermolysin on Amberlite XAD-7 in fert-amyl alcohol. In addition, Miyanaga *et al.* [17] used immobilized thermolysin in Amberlite XAD-7 for synthesis of aspartame precursor in a mixed of tert-amyl alcohol (TA) and ethyl acetate (EA) at 33:67 (v/v), the reaction was at 40°C with a yield of 99% during 3.6h. While, Kusano *et al.* [3] used free thermolysin to synthesis of N-carbobenzoxy-l-aspartyl-l-phenylalanine methyl ester (ZDFM), a precursor of aspartame. Whereas, Ogino *et al.* [25] developed the synthesis of aspartame precursor by using PST-01 protease and thermolysin, and mentioned that the rate of synthesis was slower than thermolysin as a substrate for production of aspartame precursor.

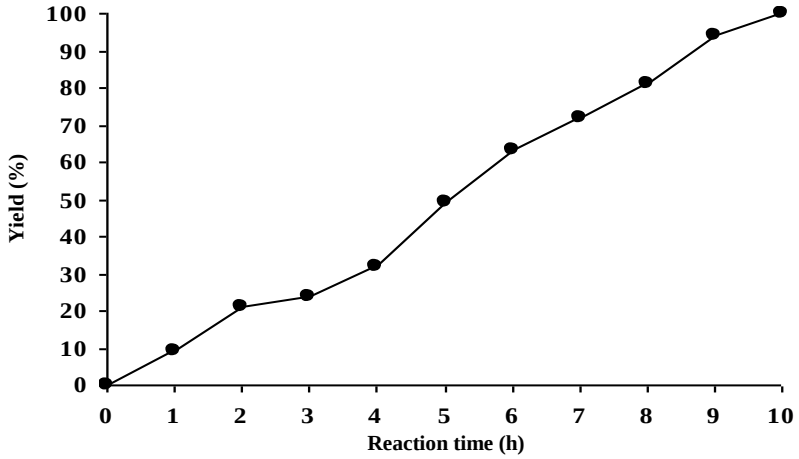

Figure 7- The yield of aspartame precursor by immobilized thermolysin on bentonite.

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