IMMOBILIZATION AND KINETICS OF AMYLOGLUCOSIDADE (AMG 200 L) ON CHITIN

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ABSTRACT

Amyloglucosidase (AMG 200 L) was immobilized on chitin polysaccharide as an inexpensive support and the kinetic parameters of immobilized form were determined. The obtained results indicated that the binding power of chitin equals to 80.37 % of the glucoamylase units added. On the other hand, the activity of bound enzyme was 89.27 % of the soluble form.

The effect of substrate concentration on reaction velocity of this enzyme pointed out that V_{max} and K_m for immobilized enzyme were 1.340 mM/1, 0.0195 M while these values for soluble form were 1.497 mM/1, 0.0143 M, respectively. The higher K_m value for immobilized enzyme may be attributed to that the binding of chitin polymer to soluble enzyme increased the affinity between the substrate and chitin-enzyme complex. The optimum pH was 4.9 for immobilized enzyme and 4.5 for the free form. The effect of temperature on enzyme activity proved that the optimum temperature for chitin-enzyme complex reached 70 °C and 60 °C for the free form. The effect of enzyme concentration illustrated that the reaction activity reached its maximum when the enzyme concentration amounted to 15×10^{-3} Lu/ ml for both forms of amyloglucosidase anzyme.

INTRODUCTION

Glucoamylase (E. C. 3.2.1.3) in soluble form is one of the most widely employed industrial enzyme. However, the use of this enzyme has been limited by difficulties such as, the problems of its high cost due to isolation and purification techniques for enzyme production, and the inability to reuse or regenerate the enzyme (Demerdash, 1978).

Soares et al. (1984) mentioned that the high cost for the production and utilization of enzymatic catalysts may be considerably diminished through enzymes immobilization on solid supports Among others, this practice provides the

following advantages: the immobilization confers the enzyme a high resistance to denaturation; it enabbles the continuous reutilization of the enzyme- support system. Also, Soares et al. (1984) in another research work stated that preliminary results with amyloglucosidase (AMG) immobilization on chitin were attractive in comparison with other supports, such as sand, glass, microsepheres, feldspar, ceramics, Merckogel, quartz, alumina, diatomite and chromosorb. Therefore, additional studies should be carried out for the optimization of chitin as a solid support for AMG immobilization. Metry (1977) mentioned that chitin is a glucan polysaccharide. Structurally it closely resembles cellulose in structure and is more insoluble and rigid with an acetamide group in place of the equatorial hydroxyl. The glycosidic linkage between the glucose units is (1e--→4e).

Schafhauser and Storey (1992) immobilized amyloglucosidase enzyme on granular chicken bone by noncovalent interactions. The amount of activity bound relative to an equal amount of free enzyme was 13.6 ± 0.4%.

Demerdash (1978) immobilized amyloglucosidase enzyme by using Amberlite IR (OH) resin. He observed that the immobilized enzyme had slightly higher K_m value than free enzyme while the optimum pH for immobilized form was 4.5 and the latter one was 5.5 - 6.5. He added that the immobilized enzyme had a narrow range of optimum temperature (65 - 70°C) while the free enzyme had a wider range (55 - 75°C). Also, Schafhauser and Storey (1992)found that the optimum temperature for free enzyme was 55°C and 70°C for immobilized form.

Rough et al. (1979) stated that the activity of this enzyme could be affected by several factors, i. e., temperature, pH, enzyme concentration, time, and dry substances. Aschengreen et al. (1979) recommended a saccharification temperature at 60°C. At temperature lower than 60°C, the activity of the enzyme was reduced and the risk of microbial infection increased. The recommended pH range by Novo (1979) with free amyloglucosidase enzyme was 4.0 - 4.5. The enzyme dosage used in the saccharification process is very important since increasing the added amount of the enzyme might lead to less amount of glucose as a net result owing to retrogradation as described by Novo (1979). In addition, the reverse reaction is catalyzed by high enzyme dosage of AMG, and therefore, it is important to add a suitable dosage of enzyme.

The aim of the present work is to immobilize amyloglucosidase enzyme on the chitin as an inexpensive support and to study the kinetic behavior of the immobilized enzyme, since these parameters are very important form the industrial and economical point of view.

MATERIALS AND METHODS

I : Glucoamylase enzyme (Amylogluosidase, AMG 200 L; E. C. 3.2.1.3. α 1→4 glucohydrolase) was supplied from Novo Laboratories, INC., Danbury, U. S. A. This enzyme was in a liquid form with an activity equals to 200 AGU / ml. (one Novo amyloglucosidase unit hydrolyses one u mole of maltose per min. at 25°C and pH 4.5).

II : Chitin support was prepared from crab shells according to the method described by Soares et al. (1984). The support was activated by using hexamethylenediamine and glutaraldehyde according to the method stated by Levy and Fusee (1976).

Immobilization of glucoamylase anzyme was carried out by the incubation of activated support with the enzyme according to the procedure described by Soares et al. (1984). The yield of AMG immobilization was determined from the amount of immobilized protein on chitin.

Protein was determined by using PIERCE BCA (Bicinchoninic acid) reagent and albumin as standard according to the method described by Smith et al. (1985). Protein assay reagents were obtained from PIERCE Company, U. S. A.

The activities of free and immobilized enzymes were determined using maltose solution according to the method described by Attia and Ali (1974).

III : Effect of different parameters

III : I : Effect of substrate concentration on rection velocity for free and immobilized amyloglucosidase enzyme (AMC 200 L)

Different concentrations of maltose solutions i. e., 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2% in acetate buffer (0.05M and pH 4.5) were used with enzyme dilution 0.15×10^{-3} I.u/1ml for free enzyme and a quantity of chitin-complex containing the same amounts of glucoamylase in the case of immobilized enzyme. The incubation period was 30 min. at 60°C. The resulting glucose was colorimetrically

determined by Somogyi method (1952). The activity was determined and expressed as reaction velocity.

III: II:Effect of pH on the activity of free and immobilized amyloglucosidase enzyme

The reaction of free and immobilized enzyme was tested at different pH values, i.e. 3.7, 4.1, 4.5, 4.9, 5.3, 5.7, 6.1, 6.5 and 6.9 using one ml of maltose soln. (1%) in acetate buffer (0.05M) with the same above-mentioned enzyme concentration and the incubation period was 30 min. at 60°C. The activity was determined by measuring the resulting glucose as mentioned before.

III :III: Effect of temperature on the activity of amyloglucosidase enzyme

The activity of free and immobilized glucoamylase enzyme at different temperatures, i.e., 30, 40, 50, 60, 70, 80, 90 and 100°C was measured using one ml. of maltose solution (1%) in acetate buffer (0.05M, pH 4.5), with the same above-mentioned enzyme concentrations. The activity was estimated as previously mentioned .

III : IV : Effect of free and immobilized amyloglucosidase enzyme (AMG 200L) concentration on reaction activity

Seven enzyme concentrations varying from 3 to 21x10^{-3} I.u/lml (free enzyme) and quantities of chitin - enzyme complex containing the same amounts of glucoamylase were used to illustrate the effect of enzyme concentration on reaction activity. The activity was determined as described by Attia and Ali (1974).

RESULTS AND DISCUSSIONS

I : Immobilization process

The immobilization process was carried out by using the method described by Soares et al. (1984), the obtained results are illustrated in table (1). These results showed that the binding power of chitin equals to 80.37%. i. e. the total units of glucoamylase bound to chitin surface was 80.37% of the glucoamylase units added. On the other hand, the activity of bound enzyme was 89.27 % of that soluble one. The decrement in the activity of immobilized enzyme may be due to that the active sites of glucoamylase are the part of the enzyme through which the enzyme is bound to the chitin, or the bound glucoamylase has a conformational alteration that affects the active sites.

In general the obtained results indicate that amyloglucosidase enzyme was immobilized on chitin. This conclusion is in good agreement with that stated by Soares et al. (1984) who mentioned that chitin revealed as a very effective support for glucoamylase immobilization through covalent bonding. This may be due to the composition of chemical structure and the high porosity of this material.

Table (1) Immobilization of amyloglucosidase enzyme on chitin

Enzyme added	Enzyme in supernatant	Bound enzyme		
		Total bound units	Bound* enzyme%	Activity of** chitin-enzyme complex
32 mg protein /1g chitin	6.25 mg protein	25.72 mg protein	80.37	89.21

* calculated as percentage from added units.

** calculated as percentage from the activity of original enzyme.

II : Effect of different parameters on the reaction velocity of immobilized amyloglucosidase (AMG 200 L) enzyme

II : I : Effect of substrate concentration

The effect of substrate concentration on reaction activity of soluble and immobilized amyloglucosidase (AMG 200L) are shown in Table (2), and Fig. (1,A). From these results. the velocity increased progressively with the increase of the substrate until it reached a maximum reaction velocity (V_{max}) for soluble form 1.497 mM / 1 and 1.340 for immobilized form. It is clear that the maximum (V_{max}) of soluble enzyme is slightly higher than immobilized enzyme. This observation can be attributed to steric effect (Handa et al., 1982).

The Michaelis constant (K_m) is one of the most useful and important parameter in understanding the behavior of enzyme (Handa, 1982), consequently, K_m for soluble and immobilized enzymes were determined and shown in Fig. (1, B). The values of K_m were 0.0143M (soluble enzyme) and 0.0195M (immobilized enzyme). The proportionally slightly higher K_m value for immobilized amyloglucosidase enzyme may be attributed to the binding of chitin polymer to soluble enzyme increased the affinity between the substrate and

Table (2) : Effect of substrate concentration on the reaction velocity of free and immobilized Amyloglucosidase (AMG 200 L) enzyme

Maltose concentration			Obtained-D-glucose mM / l		Velocity (v)		$\frac{1}{v} \times 10$	
%	$Mx10^{-4}$	$\frac{1}{S}x10^{3}$	F^*	I^*	F^*	I^*	F^*	I^*
0.2	5.84	1.712	0.406	0.284	0.058	0.390	1.704	2.567
0.4	11.70	0.855	0.689	0.427	0.113	0.076	0.883	1.318
0.6	17.54	0.570	0.841	0.632	0.164	0.111	0.612	0.904
0.8	23.39	0.428	0.975	0.726	0.210	0.144	0.475	0.697
1.0	29.24	0.342	1.134	0.910	0.254	0.175	0.394	0.572
1.2	35.09	0.285	1.213	1.045	0.295	0.204	0.291	0.489
1.4	40.94	0.244	1.328	1.129	0.352	0.232	0.284	0.430
1.6	46.78	0.214	1.416	1.274	0.369	0.259	0.271	0.386
1.8	52.63	0.190	1.497	1.340	0.403	0.285	0.248	0.351
2.0	58.48	0.171	1.440	1.340	0.435	0.309	0.230	0.324

F^* : Free enzyme I^* : Immobilized enzyme

chitin-enzyme complex, and hence higher K_m can be observed. This constant was once more determined by Lineweaver and Burk (1954) technique, as shown in Fig. (1, C). The obtained K_m values were almost equal to that obtained firstly by the half way of experimental curve.

II : II : Effect of pH

The plot of pH values versus activity of the immobilized and free enzymes may reveal the change in optimum pH of immobilized in comparison with free enzyme and this is shown in Table (3) and Fig. (2). The immobilized enzyme had a pH optimum of 4.9 with maximum activity 12.75 umM /1 /min., as compared to its soluble counterpart which had a pH optimum of 4.5 with maximum activity 13.56 umM /1/min., respectively. The obtained results are in good agreement with those obtained by Demerdash (1970) and Soares et al. (1984).

The differentiation in optimum pH values of soluble and immobilized enzymes may be due to the conformation changes in the immobilized enzyme which occurred during immobilization. Such explanation was introduced by Demerdash (1978), who immobilized glucoamylase enzyme on Amberlite IR -45-($^-$OH) resin.

It is worthy to mention that low value of optimum pH (4.5 - 4.9) clearly indicates the importance of such acid media to fit the nature of the active site in glucoamylase enzyme. Underkofler et al. (1965) stated that the formation of glucose from liquefied dextrin and maltose at lower pH reduces colour formation in the resulted glucose syrup and aids in preventing microbial contamination during the long period (48 - 72 hrs.).

III : III : Effect of temperature

Table (4) and Fig. (3) show the effect of temperature on the activity of free and immobilized amyloglucosidase (AMG 200L). The optimum temperature reached 60°C for free enzyme and 70°C for immobilized form. These obtained temperatures could be considered a balance between the increase of initial activity and destruction of the enzyme at high temperature, Plowman (1972), respectively. The slight change in behavior of free and immobilized glucoamylase can be attributed to the change in physical characteristics of immobilized enzyme. However, such values for optimum temperatures were in good agreement with that obtained by Demerdash (1978) and Schafhauser and Storey (1992).

Table (3) Effect of pH on activity of free and immobilized amyloglucosidase (AMG 200 L) enzyme

pH	Obtained - D - glucose umM / l		Reaction activity umM / l / min.	
	free enzyme	immobilized enzyme	free enzyme	immobilized enzyme
3.7	280.5	205.2	9.35	6.84
4.1	356.1	261.6	11.87	8.72
4.5	406.8	300.9	13.56	10.03
4.9	392.7	382.5	13.09	12.75
5.3	307.2	324.9	10.24	10.83
5.7	182.1	286.2	6.07	9.54
6.1	72.3	216.0	2.41	7.20
6.5	---	118.8	---	3.96
6.9	---	102.0	---	3.40

Table (4) Effect of temperature on the reaction activity of free and immobilized amyloglucosidase (AMG 200 L) enzyme

Temperature C^{O}	Obtained - D - glucose umM / l		Reaction activity umM / l / min .	
	free enzyme	immobilized enzyme	free enzyme	immobilized enzyme
30	209.70	161.4	6.99	5.38
40	530.00	390.3	17.70	13.01
50	668.7	551.7	22.29	18.39
60	740.4	657.9	24.68	21.93
70	730.2	685.2	24.34	22.84
80	547.8	432.6	18.26	14.42
90	365.7	221.4	12.19	7.38
100	252.3	123.6	8.41	4.12

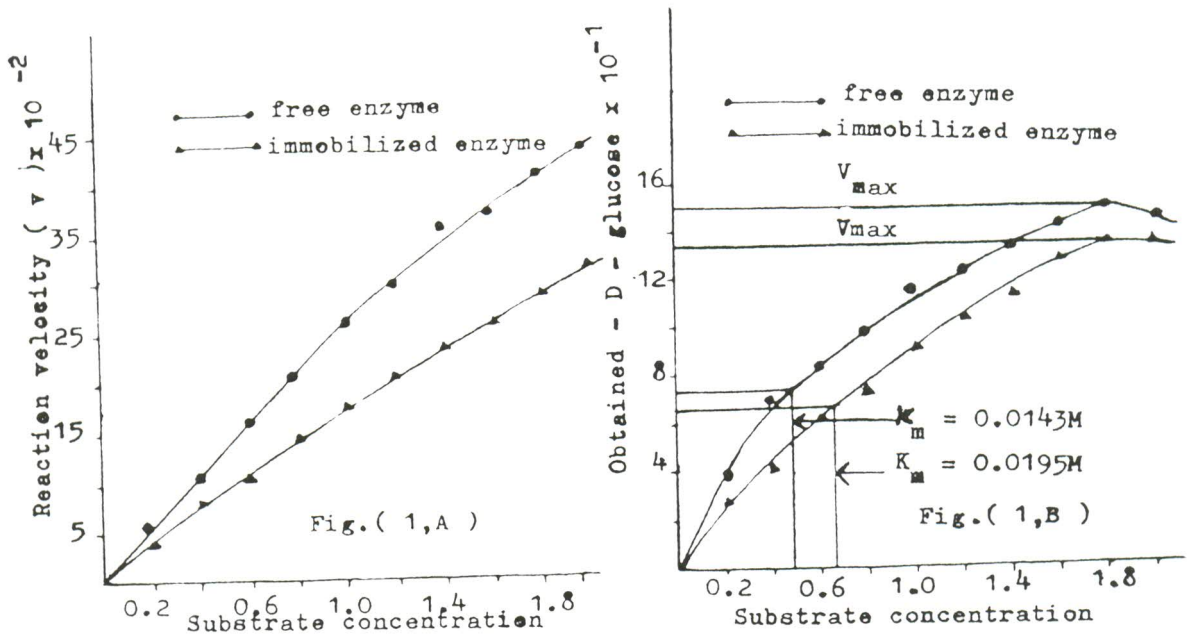

Fig. (1;A,B) Effect of substrate concentration on reaction velocity of Amyloglucosidase (AMG 200L)enzyme.

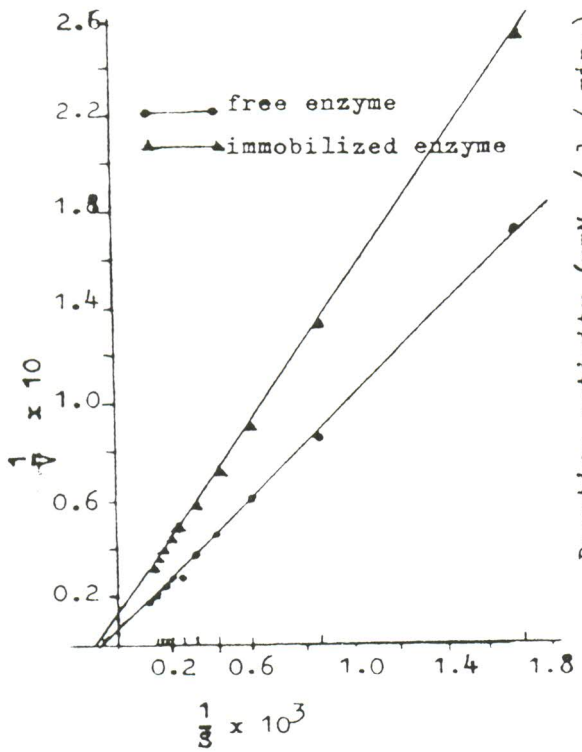

Fig. (1,C) Lineweaver - Burk plot for amyloglucosidase enzyme

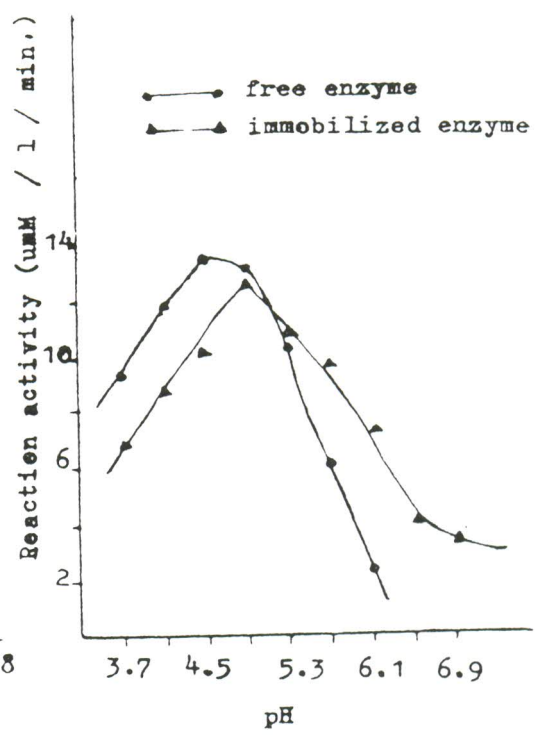

Fig. (2) Effect of pH on the reaction activity of amyloglucosidase enzyme

Table (5) : Effect of enzyme concentration on the reaction activity of glucoamylase (Amyloglucosidase AMG 200L)

Enzyme concentration I.u. x 10^{-3} / ml	Obtained-D - glucose umM / l		Reaction activity umM / l / min.	
	free enzyme	immobilized enzyme	free enzyme	immobilized enzyme
3	32.1	28.80	1.07	0.96
6	87.90	78.30	2.93	2.61
9	128.70	119.40	4.29	3.98
12	169.50	153.60	5.65	5.12
15	204.60	159.60	6.82	5.32
18	128.40	112.80	4.28	3.76
21	80.40	73.80	2.68	2.46

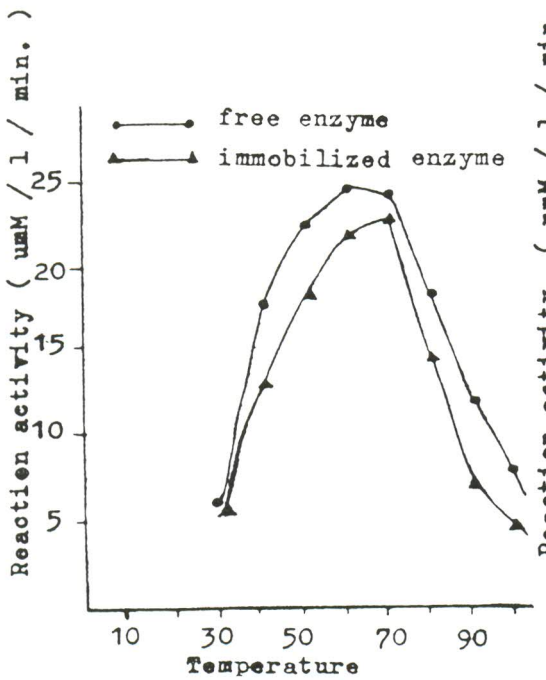

Fig. (3) Effect of temperature on the activity of amyloglucosidase enzyme

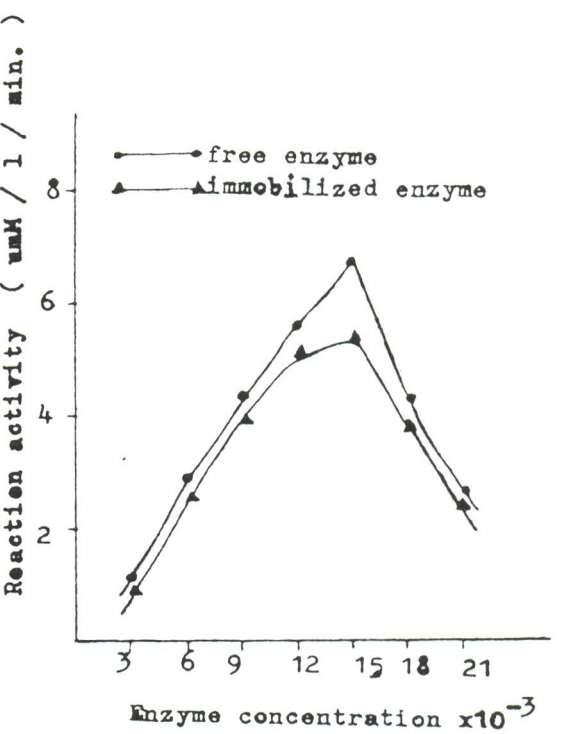

Fig. (4) Effect of enzyme concentration on reaction activity of amyloglucosidase enzyme.

III : IV : Effect of enzyme concentration on reaction activity

The obtained results are shown in Table (5) and illustrated in Fig. (4). Seven enzyme concentrations ranging between 3 to 21 I.ux10 ^{3}/1ml for soluble enzyme and quantities of chitin-enzyme complex containing the same amounts of glucoamylase. The results indicated that there were an increase in the activities for soluble and immobilized forms till enzyme concentrations 15x10 ^{3} I.u/1ml. The reaction activities reached its maximum 6.82 umM/1/min. for soluble form and 5.32 umM/min. for immobilized enzyme, then it fall down and reached 2.68 umM/1/min. and 2.46 umM/1/min. at the highest concentration i.e.21x10 ^{3} I.u/1ml. The obtained results are in agreement with that obtained by Foda (1987).

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تحميل وحركية أنزيم الاميلوجلوكوسيدير على الكيتين

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يهدف هذا البحث الى دراسة امكانية تحميل انزيم الاميلوجلوكوسيدير الذائب على الكيتين كدعامة رخيصة وكذلك دراسة السلوك الحركى لهذا الانزيم المحمل وذلك لامكانية استخدام هذا الانزيم المحمل عديد من المرات وهذه النقطة مهمة من الناحية الصناعية والاقتصادية حيث أن هذا الانزيم مهم فى عملية التسكر الخاصة بصناعة شراب الفركتوز.

أوضحت الدراسة أن قوة ربط الكيتين لانزيم الاميلوجلوكوسيدير لـ AMG 200 تساوى ٣٧ر٨٠٪ من كمية الانزيم المضافة الى الكيتين وكانت نسبة نشاط الانزيم المحمل تساوى ٢٧ر٨٩٪ مقارنة بالصورة الحرة للانزيم .

أظهرت الدراسة الحركية لهذا الانزيم أن السرعة القصوى وثابت ميكاليس لعقد(الأنزيم - الكيتين) تساوى ٣٤٥ر١. ملليمول/ لتر ، ١٩٥ر. مول أما هذه الثوابت للانزيم الحر فكانت ٤٩٧ر١ ملليمول / لتر ، ١٤٣ر. مول وقد يعزى زيادة ثابت ميكاليس للانزيم المحمل عن الانزيم الحر الى أن ارتباط الكيتين مع الانزيم أدى الى زيادة جاذبية مادة التفاعل بالانزيم.

وقد اوضحت النتائج أن درجة الحموضة المثلى pH للانزيم المحمل تساوى ٩ر٤ أما الانزيم الحر فكانت ٥ر٤ وقد اوضحت الدراسة أيضا أن درجة الحرارة المثلى لمعقد (الكيتين - الانزيم) فكانت ٧٠ درجة مئويه وللانزيم الحر تساوى ٦٠ درجة مئويه .

أما بالنسبة لدراسة تأثير تركيز الانزيم على نشاط التفاعل فقد أظهرت الدراسة أن نشاط التفاعل وصل الى اقصاه عندما كان تركيز الانزيم يساوى ١٥ × ١٠ $^{-٣}$ وحده دوليه لكلا من صورتى الانزيم.