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RESEARCH ARTICLE

Purification and Biochemical characterization of a novel Recombinant L-glutamin-(asparagin-)ase from *E.coli BL21 (DE3)*

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Abstract

The recombinant enzyme containing a 6x*Histidin*-tag can be easily purified by Ni-NTA chromatography. Besides, its molecular weight was determined to be 40 KDa by SDS-PAGE .The biochemical properties of a novel recombinant L-glutamin-(asparagin-)ase was studied in the presence of L-glutamine(L-Gln) and L- asparagine (L-Asn) as substrates. The highest activity of the enzyme has been shown at pH 8 and 7. It has also been noticed the maximum activity L-glutamin-(asparagin-)ase was o.994 and 1.006 IU/ml towards L-asparagine and L-glutamine, respectively. On the other hand, the maximum L-glutamin-(asparagin-)ase activity was at 37 and 40 °C. Moreover, the enzyme kept more than 90% of its activity at temperature degrees ranged between 25-37 °C for 30 min. Metal ions (2 and 5 mM), such as $MgSO_4.7H_2O$, $CuSO_4.5H_2O$, $FeSO_4.7H_2O$, $CaCl_2$ $2H_2O$ and KCl have a slight effect on the activity of L-glutamin-(asparagin-)ase at a high concentration of ions. Accordingly, both enzymatic activities were not modified in the presence of EDTA at 2 and 5 mM while the remaining enzyme activity % was reduced into 74% and 68% in the presence of each 5mM of 2-mercaptoethanol (2-ME) and Dithiothreitol (DTT) , respectively. The enzymatic L-glutamin-(asparagin-)ase activities increased in the presence of 5% of NaCl in the reaction buffer. Finally, the results of enzyme Kinetics have showed that the Km and Vmax values of L-glutamin-(asparagin-)ase were 0.067 mM and 0.042 mM /min towards L-asparagine and 0.054 mM and 0.041 mM/min towards L-glutamine, respectively.

INTRODUCTION

L-glutamin-(asparain-)ase is an amidohydrolase enzyme (EC 3.5.1.38) that catalyze the hydrolysis of of L-Asparagine (L-asn) and L-glutamine(L-gln) to L-aspartate (L-asp) , L-glutamate(L-glu) respectively and release ammonia (NH_3). In particular such enzymes are considered a part of the bacterial asparaginase family based on the basis of kinetic and structural studies (Ortlund *et al*., 2000). The activity of L-glutamin-(asparain-)ase enzyme occurred in a number of bacterial sources Such as *E.coli* ,*Pseudomonas sp*. , *Erwinia sp*. (Jyoti *et al*., 2011). L-glutamin-(asparain-)ase play a major role in nitrogen metabolism for both prokaryotes and eukaryotes. In the other hand the activity of L-glutamin-(asparagin-)ase can be used for different industrial and pharmaceutical purposes such as a processing aid in the manufacture of food (Kornbrust *et al* ., 2010; Sarada, 2013) and in the treatment of acute lymphoblastic leukemia (ALL) and some mast cell tumor (Huerta-Saquero *et al* ., 2013; Bülbül and Karakuş, 2013). In addition both enzymes act as a biosensor for monitoring the level of L-glutamine and L-asparagine (Sabu *et al.,* 2000).This study was aimed to study the properties of a novel recombinant L-glutamin-(asparagin-)ase from *E.coli BL21(DE3)* and this was achieved by: characterization of recombinant enzyme L-glutamin-(asparagin)ase from genetically engineered *E.coli***.**

Materials and Methods

Binding buffer (pH 7.4) ; Imidazole stock solution (2M): It was prepared by dissolving 136.16 g of imidazole in 1 litter PBS and Elution buffer 20 mM sodium phosphate, 500 mM NaCl, 10-500 mM imidazole (pH 7.4). It was prepared by dissolving 1.78 g Na_2HPO_4 .$2H_2O$, 1.38 g $NaH_2PO_4.H_2O$, 29.22 g NaCl and 1 mM β-mercaptoethanol. Add 2 M imidazole stock solution. The volume of imidazole stock solution added depends on the chosen imidazole concentration during binding

and elution. Finally, the volume was completed to 1 litter with dH_2O.

Purification of L-glutamin-(asparagin)ase

The recombinant *E.coli BL21(D3)* Cells were previously expressed *KPN-01165* gene and harvested after induction by IPTG .The supernatant was collected and used for purification. The His GraviTrapTM is a prepacked, single-use gravity-flow column containing precharged Ni Sepharose.The column was intended for purification of histidine-tagged proteins by immobilized metal affinity chromatography (IMAC).Analysis of total expression by using SDS-PAGE electrophoreses (Agostinho, 2011).

Desalting or Diafiltration

Refolded of the purified protein was done using the Amicon® Ultra-15 device, the sample, was concentrated then reconstituted to the original sample volume with wash buffer. The process of "washing out" was repeated until the concentration of the contaminating microsolute had been sufficiently reduced and the enzyme was concentrated.

Determination of Putative L-glutamin-(asparagin-)ase Activities

Dilutions were made from the ammonium sulfate stock solution to make the standard curve of ammonium sulphate at absorbance 450 nm. The activity of L-glutamin-(asparagin-)ase was assayed according to Mannan *et al*.,(1995). Using 0.04 mM L-glutamine and 0.04 mM L-asparagine as substrate.

International Unit (IU) :One international unit of Putative L-asparaginase orL-glutaminase is the amount of enzyme which liberates 1µmol of ammonia per minute per ml [µmole/ml/min].

Characterizations of Putative L-glutamin-(asparagin-)ase

A characterization of pure Putative L-glutamin-(asparagin)ase was achieved as follows:

Effect of pH on Enzyme activity (**Gaffar, 2005)**

Different pH values of substrate were run with a purified enzyme at 37˚C min for 30 min. The enzyme reaction rates for the deamidation of L-asparagine or L-glutamine at 0.04 mM of substrate (asparagine /glutamine) were determined at pH 5,6,7,8,9, and pH 10 respectively.

Effect of pH on Enzyme Stability

The purified putative L –Glutamin-(asparagin-)ase was incubated at various pH values ranging between 5 - 10 for 60 min at 37 ˚C. ,and transfer into an ice bath, then the activities of enzyme was assayed for each one and estimation the remaining activity % of enzyme.

Optimum Temperature of Enzyme Activity

The purified enzyme was incubated at different temperature values ranging between (25,30,37,40,45 and 50 °C) with 0.04M L-asparagine or L- Glutamine for 30 min. The activity was assayed at the end of reaction.

Optimum Temperature of Enzyme Stability

For enzyme stability, the purified enzyme was incubated at different temperature values (25,30,37,40,45 and 50°C) for 60 min. then transfer into an ice bath, and the activity of purified enzyme was assayed for each one and estimation the remaining activity % of enzyme.

Effect of Different Metal Salts on the Enzyme Activity.

The effects of various metal salts (2 mM and 5 mM) on L-glutaminase and L- asparaginase activity were tested by incubating an equal amount of purified enzyme with metal ion at 2 and 5 mM at 37˚ C for 30min,then the enzyme activity was assyssed after each treatment.

Effect of Reducing and Chelating Agents on Enzyme Activity

The effect of some reducing and chelating agents on purified enzyme was examined by incubating the enzyme with 2 and 5 mM of (2-mercaptoethanol, DTT, EDTA and DMSO) for 30 min,then the activities of enzyme were assayed after each treatment and estimation the remaining activity % of enzyme.

Effect of Sodium Chloride Concentrations on Enzyme Activity.

The effects of different NaCl concentrations (0, 5, 15 and 25%)on enzyme activities were studied. Asparaginase - glutaminase activities were examined by incubating for 30 min with each concentration. The activity was then assessed after each treatment.

Determination of Km and V max.

L-asparagine and L-glutamine were used in the present study as substrate in different concentrations ranging between (0.01,0.02,0.04,0.06 , 0.08 and 0.1M) The enzyme activity was monitored by using microplate reader. The reaction with each substrate was incubated at deferent time (5,10,15,20,25 and 30 min) , then the enzyme activity was assayed and calculated the Km and Vmax values.

Results

Purification and Characterization of Fusion Enzyme

Recombinant enzyme containing a 6x*Histidine*-tag can easily purified by using Ni-NTA chromatography which is based on the specific interaction between Ni^{2+} ion immobilized on a matrix and the side chains of histidine **(**De Bernardez,1998). The bands of the fusion enzyme were visible in all prepared samples around 40 KDa range (figure 1). The result showed that the His tagged enzyme was released by elution buffer under denaturing condition to increase the binding yield Because at denaturing condition the interaction of 6x*Histidin*-tag/Ni-NTA tolerates high concentrations of guanidine. Whereas under physiological conditions host proteins with histidine stretches or host proteins containing metal ions may contaminate the protein preparation. Therefore using 20 mM imidazole can be reduce this problem and enhance the solubility of the fusion protein .Ni immobilized column was used to enable binding the polyhistidine tag of the fusion enzyme. It was further used to elute the fusion enzyme by using different concentrations of imidazole. Results were then analyzed by 10% SDS-PAGE. The purified fusion enzyme band was very clear in the lane 3,and the poly histidine tag protein was then recovered in the first 4 ml of the elution buffer. The band of the purified fusion enzyme was very clear in the lanes 2 and 3.The concentration of the fusion enzyme in lane 3 was determined using bradford assay. Result showed that the concentration of the purified enzyme was 0.329 mg/ml. The best fraction that contains a clear band of the purified enzyme was collected and dialyzed against the banding buffer to remove any histidine tag that may affect the enzyme activity.

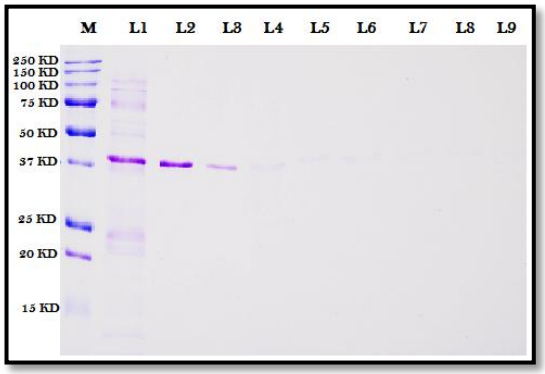

Figure (1): SDS-PAGE analysis of the purified enzyme from Bl 21 (DE3) *plysS-T1R E.coli* .M: Molecular weight of the protein marker; L1 to L9 sequential fraction of (20,40,100, 300 and 500) mM imidazole elution buffer respectively.

Refolding the Purified Enzyme by Diafiltration

Refolding can be done by desalting to remove the eluted buffer that may be interact with the activity of enzyme (De Bernardez,1998).. This property considered a distinct advantage of the diafiltration process .Then, the purified enzyme was stored at -20°C. (See figure 2).

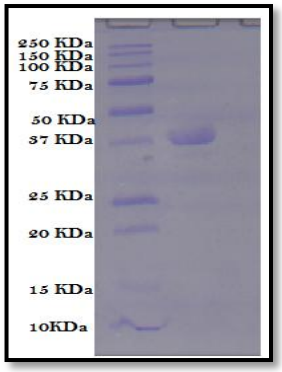

Figure (2): SDS-PAGE 10% (w/v) analysis for refolding enzyme by diafiltration. M: Molecular weight of the protein marker and L: refolded enzyme after diafiltration process.

Enzyme Characterization

The activity of the enzyme was assysed using L-asparagine and L-glutamine, as illustrated in figure (3). In the present study, the purified enzyme showed a dual activity against each of L-asparagine and L-glutamine. However, the enzyme preference was towards L-glutamine than towards L-asparagine. This was due to the interaction of the amino acid sequence of the enzyme side chain with α ammonium group of the substrate (Derst *et al*., 2000). the dual activity of this enzyme

belongs to the flexible loop of the enzyme active side that has no stable confirmation. This further resulted in the suboptimal nucleophile orientation in the active side (Aghaiypour *et al*.(2001).

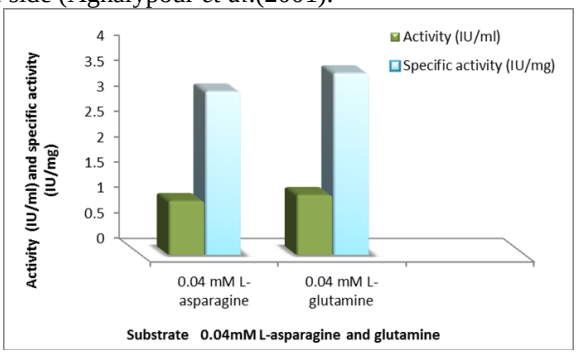

Figure (3): The activity and specificity of the enzyme toward 0.04 mM L-asparagine and L-glutamine

The Optimum pH for L-glutamin-(asparagin)ase Activity and Stability

Different pH values range from 5-10 was used to determine the optimum pH for L-asparaginase and L- glutaminase activities as in figure (4) Results shown that the highest L-glutaminase activity were 1.006 and 1.004 U/ml at pH 7 and 8 respectively, and 0.944 U/ml at pH 8 for L-asparaginase. In general ,enzymes like other protein have a limited range of pH values that kept the tertiary structure of the enzyme and its folding from denaturation and from any changes that may occurred on the its conformation(Viella and Zeikus,2001). Besides, pH values also effected on the ionization state of substrate and amino acid residues of the catalytic active site ,so any change in pH value that prevent to form an enzyme – substrate complex and undergo catalysis (Leprince and Quiquampoix, 1996). The values of pH stability for L-glutamin-(asparagin)ase was not affected at pH 7 and 8 whereas the activity of both enzymes were decreased at pH above 8 as illustrated in figure (5). Generally, the optimum pH value of microbial L- asparaginase and L-glutaminase activities are ranged between 5 to 9.

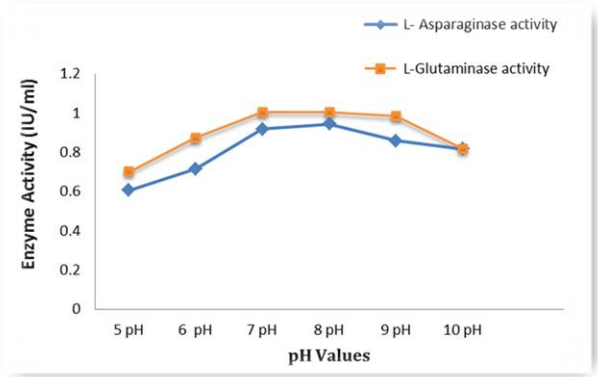

Figure (4) The optimum pH of L-glutamin-(asparagin)ase activity

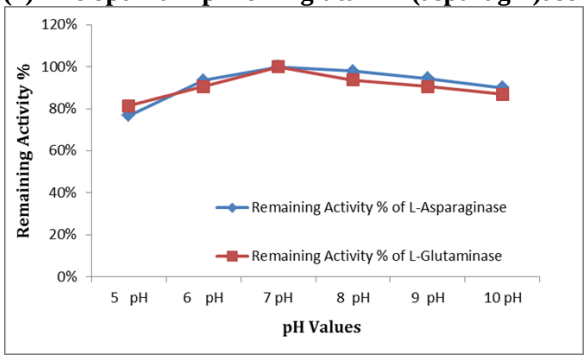

Figure (5): Stability of recombinant L-glutamin-(asparagin-)ase from *E.coli* Bl 21 (DE3) *plysS-T1R* at various pH values (5-10)

Effect of Temperature on the Activity and Stability of L-glutamin-(asparagin-)ase

The study of temperature effects on the activity of L-glutamin-(asparagin-)ase reveal that the highest activity of the optimized L-glutaminase was 1.247 U/ml at 40 °C , it was further observed that L- glutaminase was stable at 30 °C and lost its activity almost completely at 50 ◦C (figure 6).

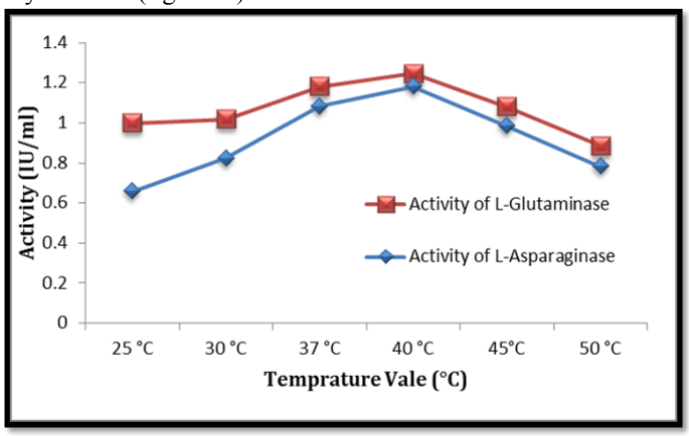

Figure (6): Effect of different temperature values (25-50 °C) on recombinant L- glutamin-(asparagin-)ase activity purified from *E.coli* BL21 (DE3) *plysS-T1R*

At the same conditions L- asparaginase activity was detected and the results shown that the best L- asparaginase activity were (1.181) and (1.08) at 37 and 40°C respectively, and the optimum temperature for enzyme stability at 30°C. This point has an advantage in industrial applications when there is need to use high temperatures. Results illustrated in figure (7) indicated that both activities were being inactivated after 30 min at 50° C. Generally, most chemical reactions required to raise the temperature degrees in order to increase the catalysis reaction of enzyme (Viella and Zeikus,2001). In the other hand the highly temperature values have an adverse effect on the activity of enzyme as a results of denaturation the three dimension structure of enzyme (Roy *et al*., 2010). Enzyme stability was determined by the primary structure of enzyme and to the some factors that may increase the stability of protein such as ions pairing, cavities, H-bond and disulfide bridges (Prescott *et al*., 2005;Roy *et al*.,2010).

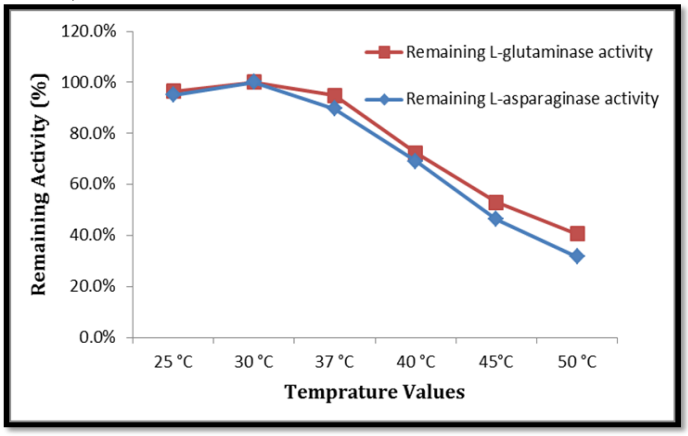

Figure (7): Thermal stability of recombinant L-glutamin-(asparagin)ase purified from *E.coli* Bl 21 (DE3) plysS-T1R

The activities of L- glutamin-(asparagin-)ase were varied in the presence of different salt levels. Results showed at 5% NaCl the enzyme was reached at the maximum activities (as shown in table 2). Generally, salt tolerance of L-glutamin-(asparagin-)ase activity played a significant role in the industrial applications that require a high level of salt environment (Madern *et al*., 2000).

Table 2 Effect of NaCl on L-glutamin-(asparagin-)ase activity

Concentration of NaCl	Remaining activity of L-glutamin-(asparagin-)ase (%)	
	L-asparaginase	L-glutaminase
0%	90	95
5%	100	100
15%	65	67
25%	37	39

Effect of Metal Ions on L-glutamin-(asparagin-)ase Activity

Activities of L-gutamin-(asparagin)ase were varied in the presence of different metal ions as indicated in table (3). The results showed that the activities of L-glutamin-(asparagin)ase were not affected by KCl and $CuSO_4$ at the 2mM and 5 mM concentrations. On the other hand, $CaCl_2$ had an inhibitory effect on both enzyme activities. It was also found the remaining activities for L-glutaminase and L- asparaginase was (92%). However L-glutamin-(asparagin-)ase activities increased in the presence of 5mM MgSO4 and $FeSO_4$ whereas no effect was seen at 2 mM.

Table (3) Effect of metal ions on the activities of recombinant L-glutamin-(asparagin-)ase purified from *E.coli* Bl 21 (DE3*) plysS-T1R*

Metal Ion	Concentration (mM)	Remaining activity of L-glutamin-(asparagine)ase (%)	
		L-asparaginase	L-glutaminase
$MgSO_4$	2mM	102	100
	5mM	105	103
$Cu\ SO_4$	2mM	97	99
	5mM	96	98
$Fe\ SO_4$	2mM	104	100
	5mM	105	105
$CaCl_2$	2mM	93	95
	5mM	92	92
KCl	2mM	98	99
	5mM	97	97

Effect of Reducing and Chelating Agents on L-glutamin-(asparagin-)ase Activity

Different reducing and chelating effect were tested with respect to the activity of L-glutamin-(asparagin-)ase. Results illustrated in table (4) revealed that the activity of L-glutamin-(asparagin-)ase was not effected with 2 and 5mM EDTA. Accordingly, it can be concluded that L-glutamin-(asparagin-)ase was not metalloenzymes. This is because it does not require metal ions for its activities (Mokrane, 2003). Results have further shown that the activity of the enzyme was lost when L-glutamin-(asparagin)ase was incubated with each of Dithiothreitol (DTT) and 2ME. This indicates that S-S bonds was possibly involved in the enzyme catalysis and had a critical role in maintaining the enzyme conformation (Kumar and Manonmani, 2013). A decrease in the microbial activity of L-glutamin-(asparagin-)ase after its treatment with a reducing agent has been investigated in many studies (Al-Naima, 2007; Zbar, 2011).

Table (4) Effect of reducing and chelating agents on the activity of recombinant L-glutamin-(asparagin-)ase purified from *E.coli* Bl 21 (DE3*) plysS-T1R*

Reducing and chelating agents	Concentration (mM)	Remaining activity of L-glutamin-(asparagin-)ase (%)	
		L-asparaginase	L-glutaminase
2- mercaptoeyhanol (2-ME)	2mM	82	79
	5mM	74	78
DTT	2mM	77	72
	5mM	68	69
EDTA	2mM	100	100
	5mM	97	100

Enzyme Kinetics

Michaelis menten parameters of L-glutamin-(asparagin-)ase were calculated from Lineweaver-Burk plot, as indicated in Table (5). The kinetic parameters of our enzyme showed that L-glutamin-(asparagin-)ase had a low Km value (0.054 mM) for L- glutamine and for L-asparagine (0.067 mM), as illustrated in figure (8). This means that L-glutamin-(asparagin-)ase had a higher affinity towards L-glutamine than L-asparagine. This was because the latter can increase the possibility of side effects during the course of anti-cancer treatment. The values of K_{cat} and K_{cat} /Km were also reported for both substrates (L- asparagine and L-glutamine). The kcat of L-glutamin-(aspargin)ase, for instance, was (127 and 124min^{-1}) whereas Km was (0.067 and (0.054 mM). At this point, the enzyme complex converts greater molecules of the substrate into a product; the reaction represents the first order rate constant.

Enzyme kinetics are a very important tool for the determination of the activity, specificity, mechanism and for the transition state structure of enzyme (Avramis, 2011).The affinities of this anti tumor enzyme to its both substrates at physiological conditions are related to its therapeutic effectiveness against tumors (kumar and Sobha, 2012).

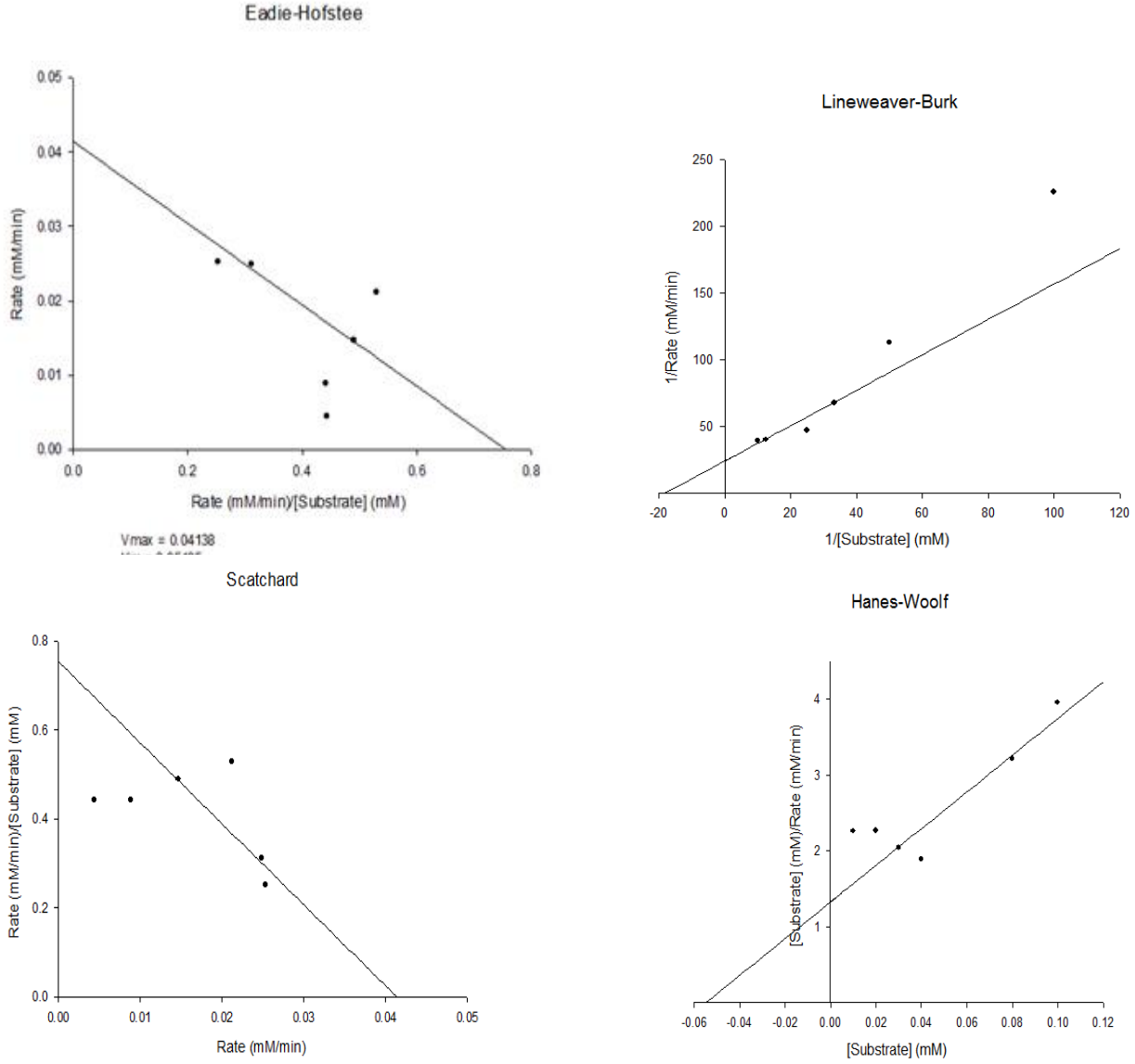

Figure (8 a) The values of Km and V max of L-glutamin-(asparagin-)ase toward L-gln as substrate toward L-gln as substrate

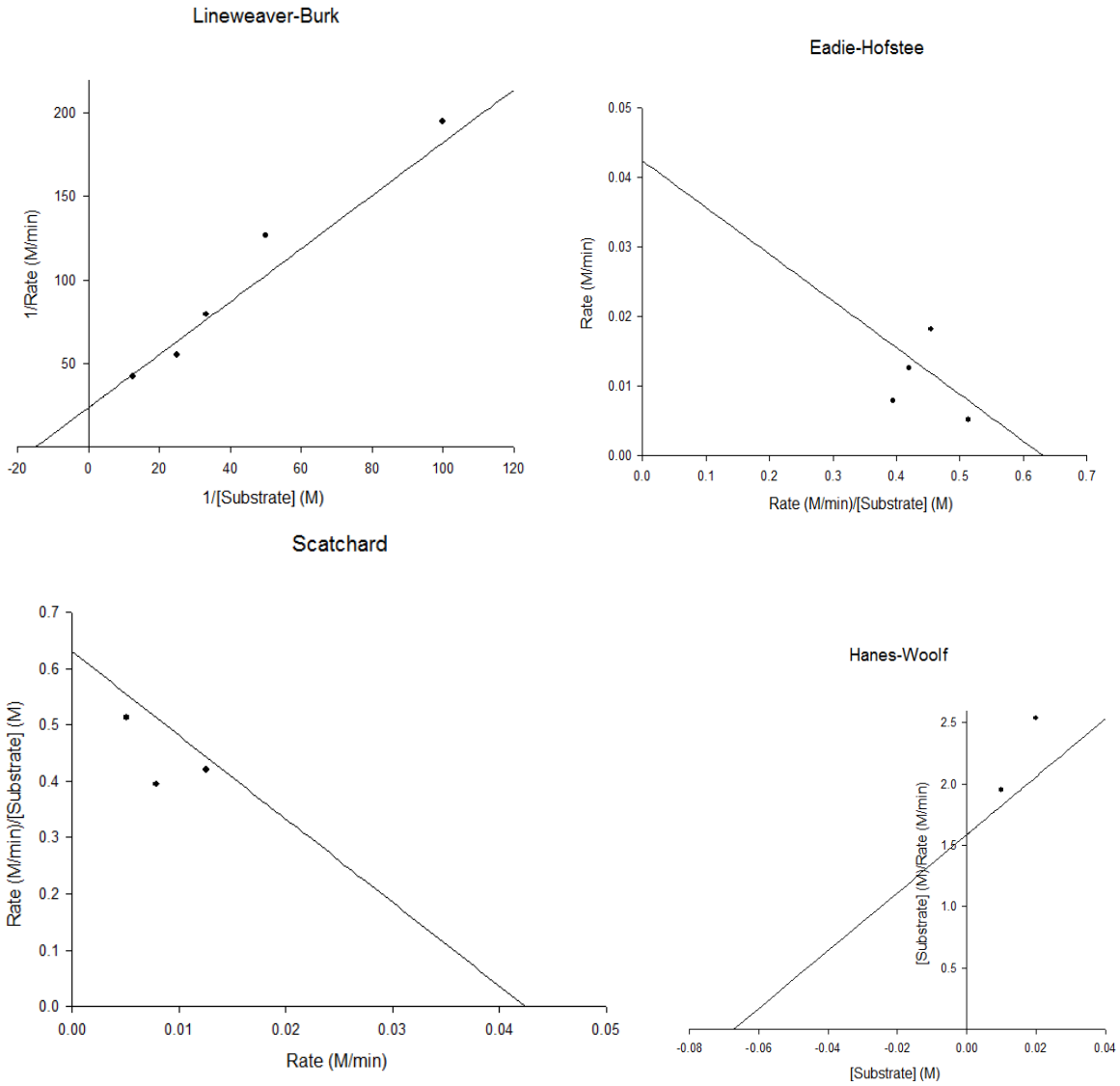

Figure (8 b) The values of Km and V max of L-glutamin-(asparagin-)ase toward L-asn as substrate

Table 5: Kinetic parameters for km, V max, K_{cat} and K_{cat} /Km the hydrolysis of L-asn and L-gln by L- glutamin-(asparagin-)ase purified from Bl 21 (DE3) *plysS-T1R E.coli* cells at 37 °C and pH 7.0

Substrate	Km (mM)	Vmax (mM/ min)	K_{cat} (min^{-1})	K_{cat}/Km (min^{-1}/mM)
L-asparagine	**0.067**	**0.042**	**0.127**	**1.89**
L-glutamine	**0.054**	**0.041**	**0.124**	**2.30**

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