Enhancement of the Activity and Stability of L-Asparaginase Food Additive Purified from *Acinetobacter Baumannii* as Anticarcinogenic in Processed Foods

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***Abstract*—**L-asparaginase is an enzyme that catalyzes the conversion of L-asparagine to L-aspartate and ammonia. The important application of the L-asparaginase enzyme in using it as food additive in processed foods for its anticarcinogenic potential. In the present study a novel strain, *Acinetobacter baumannii* S_4 isolated from sputum samples gave the highest production level of enzyme. This enzyme was purified by single chromatography step to homogeneity with a recovery yield of 25.45% and 10.58 fold of purification by using isopropanol (1:2) and CM-Sephadex C-50 chromatography. L-asparaginase was immobilized with adsorption and entrapment methods and found that adsorption by silica gel and Sephadex G-50 led to keep the activity of L-asparaginase and increased the stability of enzyme for a period of one month in comparison with other supports and this result led to increase the benefit with using L-asparaginase as food additive in processed foods such as bread, baked goods, French fries, potato chips and ready-to-eat breakfast cereals where prevents the formation of acrylamide that has carcinogenic effect and thus it leads to food safety.

***Keywords*—**L-asparaginase, *Acinetobacter baumannii*, anticarcinogenic.

I. Introduction

ACINETOBACTER genus is small aerobic Gram negative coccobacillus. *Acinetobacter* is ubiquitous free living organisms that was associated with many nosocomial infections(1).*A. baumannii* is usually introduced into a hospital by ahospitalized patient, while low percentage of infection were acquired from outside the hospital(2). The most important site of colonization is skin followed by respiratory tract and digestive tract(3). *Acinetobacter* was isolated from soil, water, fresh fruit and vegetables(2). it has an ability to survive on artificial surfaces and resist desiccation, therefore it can to diffuse in the hospital environment because their ability to form biofilms. *A baumannii* growth is suspected to be favored in hospital settings due to the constant use of antibiotics by patients in the hospital. Most *Acinetobacter baumannii* infections resulted in pneumonia, urinary tract, blood stream and surgical wound infections(4.5).

L-asparaginase catalyzes the hydrolysis of L- asparagine into L- asparagic acid and ammonia as the several types of tumor cells require(2). L- asparagine is an essential amino acid for protein synthesis(6). It has received increased attention in recent years for its anticarcinogenic potential. L-asparagine is a precursor of acrylamide. In the process of browning that occurs during the baking, frying and grilling of products made from cereal or potato acrylamides are formed at temperatures higher than 100ºC from amino acids and component of sugar. Under specific cooking conditions, L-asparagine can react with certain carbohydrates in the food to form acrylamide, a potential human carcinogen and harmful to genes(7,8). Through the addition of the enzyme L-asparaginase, the production of L-asparagine during cooking can be suppressed and the acrylamide contane may be reduced by up to ninety percent. The nutritional value, taste and browning of the product remain unaffected. L-asparaginase was used as food additive in foods such as bread, white and whole wheat flour, baked goods, French fries, potato chips, ready-to-eat breakfast cereals, and certain types of flavors called reaction flavors, since L-asparaginase is only active during food processing and is inactivated by heat and digestion. Therefore, it is very unlikely that the consumption of L-asparaginase would cause an allergic reaction(9,10).

The L-asparaginase is found in many animal and plants tissues, bacteria, and in the serum of certain rodents but not in mankind and the microbial L-asparaginase has received a great attention(11,12). L-asparaginase is produced by a large number of microorganisms such as *Enterobacter, Aerobacter, Bacillus, Erwinia, Pseudomonas, Serratia, Xanthomonas, Photobacterium, Vibrio* and *Proteus*(11,13). In addition, many fungi, yeast, actionomycetes and algae are efficient producer of L-asparaginase, therefore; there is a continuing need to obtain newer sources to increase the yield of L-asparaginase productivity . In the present study, we investigated L-asparaginase production by *Acinetobacter baumannii* in addition to purification and immobilization this enzyme to increase the stability.

II. Material And Methods

Sample collection

The study included a total of 80 samples of which 40 were from sputum and 40 were from blood collected from patients at different hospitals in Baghdad city.

Bacteriological analysis

The collected microbial sources were transported to the laboratory. Samples were plated primarily on to blood agar and MacConkey agar which was incubated at 37ºC for 24 hour the bacterial isolates were observed for morphological characters and identified by using the tests guided by Berge's Manual of systemic bacteriology. Suspicious isolates were presumptively identified by using colony morphology, negative oxidase test, positive catalase test inability to motile coccobacilli (14). Further, the *Acinetobacter baumannii* isolate was confirmed by using API 20E biochemical kit and the Vitek 2 system by using Vitek GNI card (bio Mérieux, France) according to the manufacturer's instructions.

Screening for L-asparaginase production

Semi-quantitative analysis

All bacterial isolates were evaluated for their ability to produce L-asparaginase by growing on agar based M9 medium composition for 1 litre (6.0g $Na_2HPO_4.2H_2O$; 3.0g KH_2PO_4 ; 0.5g NaCL ; 2.0g L-asparagine , 0.5g $MgSO_4.7H2O$; 0.014g $CaCl_2.2H_2O$;2.0 lactose (w/v) and 20g agar) pH= 7.0 supplemented with phenol red (few drops)as an indicator. The inoculated agar plates were incubated at 37ºC in an incubator .Pink zone radius and colony diameter was measured from positive isolates after incubation for 24 hour (15).

Quantitative analysis

The selected bacterial isolates were grown in M9 medium without addition of agar and incubated on the rotary shaker at 150 rpm at 37ºC for 24h. After removal of cells by centrifugation at 8000 xg for 25 min, the clear supernatant was used as the crude extracellular enzyme source and the amount of L-asparaginase produced was assayed.

L-asparaginase assay

The enzyme was assayed by direct nesslerization method according to the method of (16) as follows: 0.1 ml enzyme preparation was added to 1.7 ml of 0.5 M Tris-HCl buffer (pH 7.5) and the reaction was initialed by adding 0.2 ml of 0.1 M L-asparagine for 30 min. at 37ºC. Reaction was stopped by the addition of 0.5 ml of 1.5 M trichloro-acetic acid and precipitated protein was removed by cooling centrifugation at 8000 xg for 10 min. The reaction mixture was diluted to 6.5 ml with deionized water and 0.5 ml of nessler reagent was added , and kept for 15 min at 20ºC before estimation of ammonia by determination the OD at 500 nm. Standard curve was prepared with ammonium sulfate. One L-asparaginase unit (IU) is defined as that amount of enzyme, which liberates 1 µM of ammonia / min. under the optimal assay conditions.

Estimation of protein content

The protein content of the enzyme was determined by using Bradford dye method with BSA as a standard (17).

Purification of L-asparaginase

Acinetobacter baumannii cells were grown at 37°C in shaker incubator for 18-24h in M9 broth medium. *Acinetobacter baumannii* extracellular L-asparaginase was purified by using the method that described by Muslim(18). After centrifugation at 10000xg for 30 min at 4°C the supernatant was carefully removed and L-asparaginase activity in supernatant (crude enzyme) was assayed. L-asparaginase was purified to homogeneity by chilled Isopropanol (1:2) precipitation and column chromatography. The partial purification of enzyme was done by adding chilled ethanol(1:2) to crude enzyme and keeping it for overnight incubation. The precipitates were collected by centrifugation at 9000 xg for 15 min, dissolved in 5 ml of 50 mM tris-HCl buffer, pH 7.5 and dialyzed overnight against the same buffer. The dialyzed fraction was applied to the column of CM Sephadex C-50 that was pre-equilibrated with 50mM tris-HCl buffer, pH 7.5. It was eluted with NaCl gradient (0.1-0.5M) and 0.1M borate buffer, pH 7.0. The active fractions (3ml/tube) were collected then L-asparaginase activity and protein concentration were measured.

Immobilzation of L-asparaginase

1-Immobilzation by entrapment

Entrapment in calicium alginate

One ml of enzyme was mixed with 10 ml of 5% (w/v) Na-alginate. The mixture was extruded drop wise through a sterile syringe(10 ml) in to 2% cold $CaCl_2$ solution to obtain small beads with 1 mm diameter and kept for 1 hour at 4ºC. The resulting beads were collected and washed with distilled water to remove the unbound enzyme. One gram was transferred to test tube and 2ml of L-asparagine solution was added then incubated at 37ºCfor 10 min and enzyme activity was measured(19).

Entrapment in agar- agar

Two ml of enzyme was mixed with 25 ml of sterile agar-agar solution, shacked well for few minutes and poured into petri dish and allow to solidify after solidification, cubes(1x1x1 cm) were cut and washed with d.w. L-asparaginase activity was measured by addition of 1 gm agar cubes to 2 ml of L-asparagine and kept for 10 min at 37ºC then enzyme activity was measured and repeated every week for one month(19).

2- Immobilzation by adsorption

Immobilization of L-asparaginase by adsorption was carried out with different carriers including silica gel, egg shell, glass beads, aluminium foil, plastic particles, DEAE-Cellulose and DEAE-Sephadex G-50.One ml of enzyme was mixed with 1 gram of each carrier and incubated at 37ºC for 24 hour. After incubation the carriers were filtered by Waatman no.1 and washed several times with normal saline to remove non- bound enzyme. About 0.5 gm of immobilized enzyme was mixed with 2 ml of L-asparagine solution then L-asparagine activity was measured and repeated every week for one month(19).

III. Results And Discussion

Isolation of *Acinetobacter baumannii*

Six(7.5%) *Acinetobacter baumannii* isolates was isolated out of 80 collected samples. Among which 4/6 (67%) were

from sputum, 2/6(33%) were from blood(figure-1). *Acinetobacter baumannii* was isolated from sputum in high rate because their association with lower respiratory tract infections(20). *Acinetobacter baumannii* was also isolated from blood, this reflex the fact of their association with other infections(3). *Acinetobacter* spp. isolates are mostly lead to various nosocomial infections like respiratory tract infections, blood stream infections, wound infections and urinary tract infections(21). Among all nosocomial bacterial isolates, *Acinetobacter* spp. form16.9%(22). The ability of *Acinetobacter* to colonize hospital equipment and adhesion to artificial surfaces for a long periods of time reach to several months was related with biofilm formation(21). In a study done by(23) found that *Acinetobacter baumannii* was the most frequently species in clinical sources, followed by other species of *Acinetobacter*.

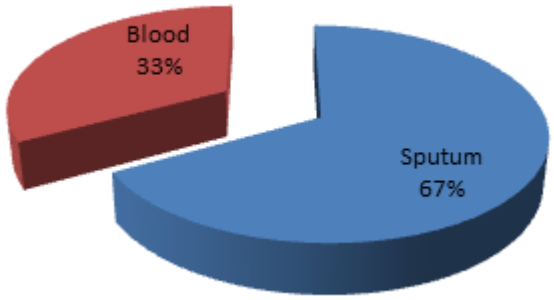

Fig. 1 Percentage for isolation of *Acinetobacter baumannii* from different clinical sources

Screening for L-asparaginase production

1-Semi-quantitative analysis

Six *Acinetobacter baumannii* isolates could grow on M9 medium supplemented with agar and phenol red since the color was change from yellow to pink which revealed the alteration in conditions from acidic to alkaline by accumulation of ammonia in the medium. Among these isolates 4 isolates showed pink zone around colonies and 2 isolates revealed pink zone within colonies(table-1). *Acinetobacter baumannii* S_4 gave higher productivity since it exhibited the largest red zone incomparison with their colony diameter. This Semi-quantitative analysis is easy to screen for L-asparaginase producers by examining the plate color

TABLE I
COLONY DIAMETER ZONE REDII AND L-ASPARAGINASE ACTIVITIES OF *ACINETOBACTER BAUMANNII* ISOLATES.

Isolate No.	Colony diameter (Cm)	Zone radius (Cm)	Enzyme unit (Unit/ml)
S1	1.3	0.2	2.47
S2	1.4	0.3	6.61
S3	1.7	0.5	13.11
S4	1.3	0.8	13.53
S5	1.0	0.4	4.98
S6	1.5	0.7	9.13

2-Quantitative analysis

All *Acinetobacter baumannii* isolates that showed positive results in semi-quantitative analysis either pink zone around or within colonies were tested for L-asparaginase production in M9 medium supplemented with phenol red by Nesslerization. The productivity of these isolates to L-asparaginase enzyme ranged from 2.47 to 13.53 Unit/ml(table-1). *Acinetobacter baumannii* S_4 also showed high level of L-asparaginase activity reached to 13.53 Unit/ml. There are many bacterial genera that can produce of L-asparaginase in different levels such as

E.coli, *Serratia* spp., *Pseudomonas aeruginosa*, *Bacillus* spp., *Aeromonas* species and *Proteus* spp. *E.coli*, *Erwinia aroideae* and *Pseudomonas flourescens*(24). The production of L-asparaginase by *E. coli*, isolated from sewage enzyme was carried out by using L-asparagine as a substrate(6).

Purification of L-asparaginase

L-asparaginase was purified by two steps of purification, the first step involved the precipitation with Isopropanol solvent at ratio(1 supernatant: 2 solvent) this solvent led to precipitate of L-asparaginase with specific activity of 6.41U/mg and fold of L-asparaginase purification of 4.0 with a yield of 31.23% (table-2). The second step of purification was included ion exchange chromatography by using CM-Sephadex C-50 column, since the precipitated protein in the first step was applied to this column and eluted with 0.1 to 0.5M NaCl gradient. The elution led to appear three protein peaks and the L-asparaginase activity was located in the third protein peak. This step led to increase the specific activity to 16.94 U/mg protein with 10.58 fold and a yield of about 25.45%(Figure-2).

TABLE II
SUMMARY OF L-ASPARAGINASE PURIFICATION FROM *ACINETOBACTER BAUMANNII*

Step	Size (ml)	Total Protein (mg/ml)	L-asparaginase activity (U/ml)	Specific activity (U/mg)	Total activity	Fold of purification	Yield (%)
Crude supernatant	100	8.42	13.53	1.60	1353	1	100
Isopropanol precipitation	16	4.12	26.41	6.41	422.56	4.0	31.23
CM-Sephadex C-50	8	2.54	43.05	16.94	344.4	10.58	25.45

L-asparaginase was purified from *Erwinia carotovora* by sulphopropyl sephadex chromatography with 4.7 fold purification and 24% a yield(25). L-asparaginase produced by *E. coli* was partially purified by ammonium sulphate precipitation20-40% with 2.15 fold of purification and 20% yield(11). In addition, L-asparaginase was purified from *Pseudomonas aeroginosa* by sephadex G-100 gel filtration(6).

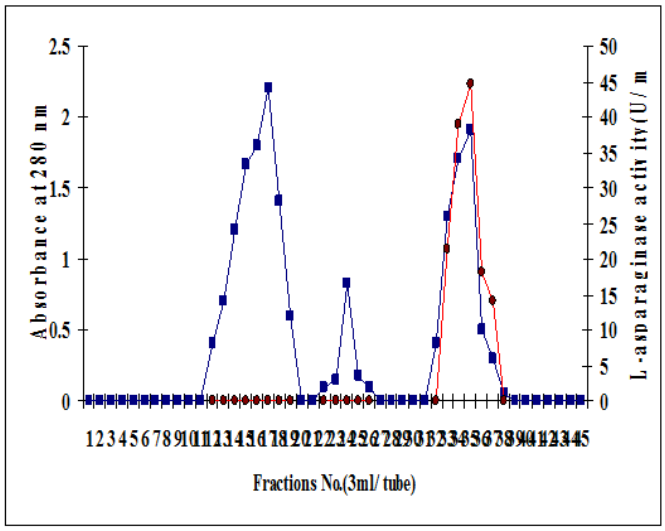

Fig. 2 Purification of L-asparaginase from *Acinetobacter baumannii* S_4 using ion exchange on CM Sephadex C-50. (●) refer to protein, (▲) refer to L-asparaginase activity

Immobilzation of L-asparaginase

1-Immobilzation by entrapment

Immobilization of L-asparaginase by entrapment also gave good results, a few decrease in L-asparaginase activity was observed and the remaining activity of immobilized L-asparaginase was 96% with agar- agar entrapment and the activity was kept for two weeks, whereas it was 94% of calcium alginate(figure-3) and was kept for two weeks(table-3). There are two counteracting affecting the efficiency of the immobilization by entrapment, the diffusion efficiency and enzyme leak out. Higher concentrations of alginate reduce the pore size of beads and consequently, reduce leakage of enzyme. However, the diffusion efficiency of the substrate and transport also reduce. A lower percentage of alginate increase the pores size, which increases leakage of enzyme, and thus decrease the remaining activity as explained by(26). In general it can be say that immobilization of L-asparaginase by adsorption more efficient than entrapment method.

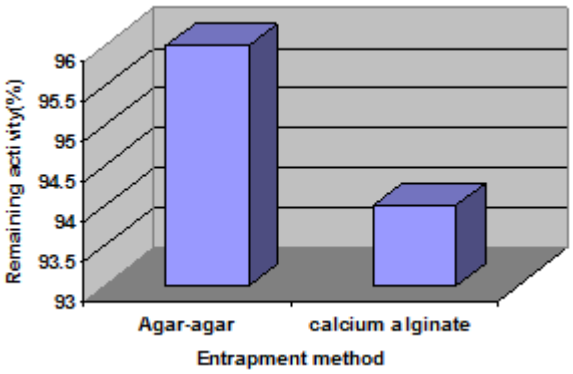

Fig. 3 The remaining activity of *Acinetobacter baumannii* L-asparaginase immobilized by entrapment

2-Immobilzation by adsorption

L-asparaginase was adsorbed on many solid supports included sand , silica gel, white egg shells, glass beads, aluminum foil, plastic pieces, DEAE-Cellulose and Sephadex G-50. These materials were able to retain high L-asparaginase activity(figure-4) and the remaining activity was high and ranging between 97-100%. The immobilized L-asparaginase by adsorption on silica gel and Sephadex G-50 had the highest remaining activity(100% and 99.5%, respectively). These two materials led to keep the activity for a month and DEAE-Cellulose kept the activity for 3 weeks while the other materials led to decrease the activity(table-3). This may be due to their hydrophilic character and great number of hydroxyl groups on the surface capable of chemical reaction, the immobilization of enzyme on solid supports may take place by ionic interactions and depends on the changes of protein(enzyme) and support such as ionic exchangers(27). According to these results we can conclude that The immobilized L-asparaginase was found to be stable during continuous operation for one month and to be resistant to attack by proteolytic enzymes, this led to enhance the activity and stability of L-asparaginase and increasing the benefit from using it as food additive in foods such as bread, white and whole wheat flour, baked goods, French fries, potato chips, ready-to-eat breakfast cereals where prevent the formation of acrylamide in processed foods that has carcinogenic effect and led to food safety

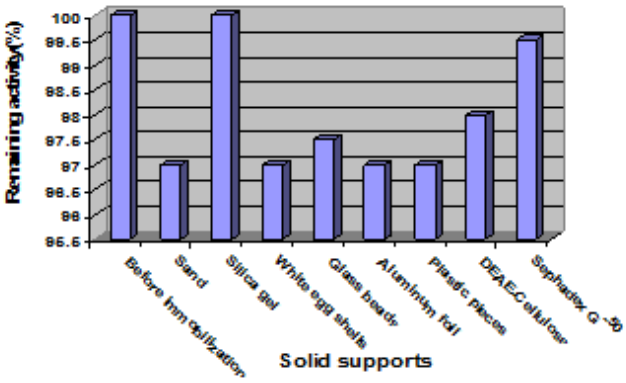

Fig. 4 The remaining activity of *Acinetobacter baumannii* L-asparaginase immobilized by adsorption on different materials

TABLE III
THE REMAINING ACTIVITY OF *ACINETOBACTER BAUMANNII* L-ASPARAGINASE IMMOBILIZED BY DIFFERENT SUPPORTS DURING ONE MONTH

Support	Remaining activity(%)			
	First week	Second week	Third week	Fourth week
Agar- agar	96	96	73	44
Calcium alginate	94	94	56	32
Sand	97	52	46	18
Silica gel	100	100	100	100
White egg shell	97	74	70	64
Glass beads	97.5	90	81	76
Aluminum foil	97	79	63	22
Plastic pieces	97	67	33	09
DEAE-Cellulose	98	98	98	89
Sephadex G-50	99.5	99	99	99

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