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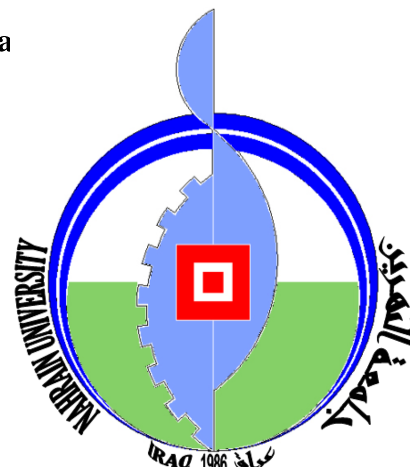

Extraction and Purification of Asparaginase enzyme from *Pisum sativum* plant and studying their cytotoxicity against L20B tumor cell line

A Thesis

Submitted to the College of Science Al-Nahrain University as a Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

By

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Acknowledgments

First of all Praise to Allah the lord of the universe, peace be upon Mohammed the messenger of Allah and upon his Relatives.

Great thank for my supervisor Dr. Nabil Khalaf for his support throughout my study. My endless thanks go to my supervisor Dr. Hameed Majeed for his kind follow up through out the research stages. I specially appreciate his warm and gentle approach to my supervision, as well as his immense patience in all his dealings with me.

My special thanks with well-beloved to the wonderful woman, Mrs. Ayat Adnan. The knowledge I gained from her and both academic and non-academic matters have been invaluable and will definitely be beneficial to my future career.

A word of thanks is due to Professor Dr. Kadim Ibrahim for his encourgment in my whole universal journey. Also it is a pleasure to thank Miss. Raghad Kadhim (my soil sister) and Dr. Qais Majeed for their kindness and help.

My sincere thanks and appreciation go to Dr. Majid Hanshel which classified the studied plant and to Dr. Farooq Ibrahim which gave me a hand to complete this work.

This is an opportunity to thank all staff and employer of Biotechnology researches center at Al-Nahrain University for all kinds of help and facilities they offered me to accomplish this work especially Dr.Kalid Abas, Dr.Ibrahim Jomaa, Dr.Hazim Al-Ahmed, and all members of microbiology department.

I am grateful to my dear Mrs. Faton Ali to her kind friendship, help and endless understanding and to all my colleges. Deep thanks to my special teacher Dr.Shahla Jasep and to Mr. Abd Al-Majeed Modafer, Mr. Zaid Nsaif, Mrs.Sabah Mahdi, Dr. Rawaa Mohammed and Mrs. Lamees Ahmed, Dr. Wafaa Gazi, Miss Boshra Abd Al-kader and Mrs.Farah Thamer. I would like to thank my family, and a grateful thank to my sister Noor for her moral love, thank you very much.

Finally heartful gratitude to the women who spent the night to comfort me, to whom the paradise be under her feet to my deer and tenderhearted, my mother.

Yours Truly, Zena Abdullah.

Summary

Plant samples of *Pisum sativum* were collected from crop fields in the Collage of Agriculture/ University of Baghdad and were classified as *Pisum sativum* subspp. *Jof* according to their morphological characteristics. Activity of asparaginase was detected in seeds, stems and leaves extracts. Results showed that maximum asparaginase activity was detected in seeds extracts which was 30.0 U/mg in comparison with 26.4 and 16.1 U/mg in extracts of leaves and stems respectively. According to these results plant seeds were used as a source for asparaginase production, characterization, and studying its antitumor activity.

Optimum conditions for the activity of crude asparaginase extracted from plants seeds were studied. Results showed maximum activity of asparaginase was achieved when the enzyme was incubated with 200mM of asparagines in a ratio of 1:3 (V/V) at 37°C for 30 minutes in presence of 0.05 M of potassium phosphate buffer solution at pH8.

Crude asparaginase extracted from plant seeds was purified in two steps, ion exchange chromatography by DEAE-Cellulose and gel filtration chromatography by Sephadex G-200. Specific activity of purified asparaginase was 228.8 U/mg.

Asparaginase purified from seeds extracts was then characterized. Results of characterization showed that the molecular weight of asparaginase was 66,464 Kelo dalton, and the optimum pH for enzyme activity and stability was pH 8.5, while the optimum temperature for enzyme activity and stability was 37°C and 40°C respectively. On the other hand the enzyme

activation energy was 6260 calories/mol, and the temperature coefficient (Q_{10}) for asparaginase was 1.32.

Antitumor activity for the purified asparaginase was studied using L20B tumor cell line by incubation with gradual concentration of purified asparaginase for 48 hours. Results showed that asparaginase extracted and purified from seeds of *P. sativum* has inhibitory effect on L20B tumor cell line.

List of Contents

Item No.		Page No.
	Summary	I
	List of Contents	III
	List of Tables	VIII
	List of Figures	IX
	List of Abbreviations	XI
Chapter one: Introduction and Literature Review		
1.	Introduction and Literature Review	1
1.1	Introduction	1
1.2	Literature Review	4
1.2.1	*Pisum sativum*	4
1.2.2	Classification of *Pisum sativum* (Pea)	5
1.2.3	Nutritional value of *Pisum sativum*	5
1.2.4	Aspraginase	6
1.2.5	Asparaginase hydrolysis	7
1.2.6	Asparaginase families	7
1.2.7	Distribution and occurrence of asparaginase	8
1.2.8	Plant asparaginase	10
1.2.9	General mechanism of the reaction catalyzed by asparaginase	11
1.2.10	Molecular structure of plant-type asparaginase	12
1.2.11	Subcellular Localization of asparaginase	14
1.2.12	Application of asparaginase	14
1.2.13	Optimum condition for asparaginase production	15
1.2.13.1	Substrate concentration	16

1.2.13.2	Reaction time	16
1.2.13.3	pH	16
1.2.13.4	Temperature	17
1.2.13.5	Buffer	17
1.2.13.6	Enzyme:substrate ratio	18
1.2.14	Purification of asparaginase	19
1.2.15	Characterization of asparaginase	19
1.2.15.1	Molecular weight determination of asparaginase	19
1.2.15.2	Optimum pH for asparaginase activity and stability	19
1.2.15.3	Optimum temperature for asparaginase activity and stability	20
1.2.15.4	Enzyme Specificity	20
1.2.16	Asparaginase: a promising chemotherapeutic agent	21
1.2.17	Leukemic treatment with asparaginase	23
Chapter two: Materials and Methods		
2.	Materials and Methods	25
2.1	Materials	25
2.1.1	Equipments and Apparatus	25
2.1.2	Chemicals and Biological Materials	26
2.1.3	Solutions, buffers and indicators	27
2.1.3.1	Nessler's Reagent	27
2.1.3.2	Potassium phosphate buffer	27
2.1.3.3	L-asparagine solution	28
2.1.3.4	Ammonium sulfate stock solution	28
2.1.3.5	Trichloroacetic acid solution	28
2.1.3.6	Bovine Serum Albumin stock solution	28

2.1.3.7	Coomassie brilliant blue G-250	29
2.1.3.8	Sodium Hydroxide solution	29
2.1.3.9	Ethylenediaminetetraacetic acid	29
2.1.3.10	Glycerol	29
2.1.3.11	Sodium chloride solution	29
2.1.3.12	Phenylmethylsulfonylfluoride	29
2.1.3.13	Sodium hydroxide solution	30
2.1.3.14	Hydrochloric acid solution	30
2.1.3.15	Sodium chloride solution	30
2.1.3.16	Potassium phosphate buffer	30
2.1.3.17	Blue Dextran solution	30
2.1.3.18	Phosphate buffered saline	31
2.1.3.19	Trypsin solution	31
2.1.3.20	Versine solution	31
2.1.3.21	Trypsin-Versine solution	31
2.1.3.22	Neutral Red solution	31
2.1.3.23	Destaining Buffer solution	31
2.1.3.24	Trypan Blue solution	32
2.1.3.25	Antibiotic solutions	32
2.1.4	Media	32
2.1.4.1	RPMI-1640 Cell line Growth medium	32
2.1.4.2	Maintenance medium	33
2.1.4.3	Cell line	33
2.2	Methods	33
2.2.1	Method of Sterilization	35
2.2.1.1	Moist heat sterilization (Autoclaving)	35
2.2.1.2	Dry heat sterilization	35

2.2.1.3	Membrane sterilization (Filtration)	35
2.2.2	Sample collection	35
2.2.3	Classification of *Pisum sativum*	35
2.2.4	Extraction of asparaginase	35
2.2.5	Enzyme assay	36
2.2.5.1	Determination of ammonia concentration	36
2.2.5.2	Ammonium sulfate standard solutions	36
2.2.5.3	Standard curve of ammonium sulfate	37
2.2.5.4	Determination of asparaginase activity	38
2.2.6	Determination of Protein concentration	39
2.2.6.1	Preparation of Bovine Serum Albumin stock solution	39
2.2.6.2	Standard curve of bovine serum albumin	40
2.2.7	Determination of optimum conditions for crude asparaginase activity	41
2.2.7.1	Effect of substrate concentration	41
2.5.7.2	Effect of reaction time	41
2.2.7.3	Effect of pH	41
2.2.7.4	Effect of Temperature	41
2.2.7.5	Effect of buffer pH	42
2.2.7.6	Effect of Enzyme:substrate ratio	42
2.2.8	Purification of asparaginase	42
2.2.8.1	Precipitation with ammonium sulfate	42
2.2.8.2	Dialysis of enzyme	43
2.2.8.3	Purification by Ion exchange chromatography	43
2.2.8.3.A	DEAE-Cellulose Preparation	43
2.2.8.3.B	Sample loading	44

2.2.8.4	Gel filtration chromatography	44
2.2.8.4 A	Preparation of Sephadex G-200 column	44
2.2.8.4.B	Sample loading	44
2.2.9	Characterization of purified asparaginase	45
2.2.9.1	Determination of Molecular Weight	45
2.2.9.2	Determination of optimal pH for asparaginase activity	46
2.2.9.3	Determination of optimal pH for enzyme stability	46
2.2.9.4	Determination of optimal temperature for enzyme activity	46
2.2.9.5	Determination of Activation Energy (Ea)	47
2.2.9.6	Determine the optimal temperature for enzyme stability	47
2.2.9.7	Determination of Enzyme specificity	48
2.2.10	Antitumor activity of purified aparaginase	48
2.2.10.1	Subculture of L20B tumor cell line	48
2.2.10.2	Detection of Cell Growth Inhibition	48
Chapter three: Results and Discussion		
3.	Results and Discussion	50
3.1	Collection and Classification of *Pisum sativum*	50
3.2	Detection of asparaginase in plant parts of *Pisum sativum*	51
3.3	Optimum conditions for asparaginase activity	53
3.3.1	Effects of substrate concentration	54
3.3.2	Effect of reaction time	56
3.3.3	Effect of buffer pH	57
3.3.4	Effect of the reaction temperature	58

3.3.5	Effect of buffer solution	60
3.3.6	Effect of enzyme:substrate ratio	61
3.4	Purification of asparaginase extracted from seeds of *Pisum sativum*	63
3.4.1	Ion exchange chromatography	64
3.4.2	Purification using gel filtration chromatography	67
3.5	Characterization of purified asparaginase	70
3.5.1	Molecular weight of asparaginase	70
3.5.2	Optimum pH for enzyme activity	71
3.5.3	Optimum pH for asparaginase stability	73
3.5.4	Optimum temperature on asparaginase activity	74
3.5.5	Optimum temperature for stability asparaginase	76
3.5.6	Activation energy and Temperture Coefficient (Q_{10}) of asparaginase	77
3.5.7	Enzyme specificity against different substrate	79
3.6	Cytotoxicity of asparaginase on tumor cell line	81

List of Table

Table No.	Subject	Page No.
(1-1)	The main Organisms that produce asparaginase with its antitumor properties	9
(2-1)	preparation of ammonium sulfate for standard curve of ammonium sulfate.	
(2-2)	Standard proteins used for the determination of asparaginase molecular weight	45
(3-1)	Morphological characteristics of *Pisum sativum* sample	51
(3-2)	Asparaginase activity and specific activity in the extract of plant parts of *Pisum sativum*	53
(3-3)	Activity of asparaginase extracted from *Pisum sativum* seeds after precipitation with ammonium sulfate using different saturation ratios	64
(3-4)	Purification steps of asparaginase extracted from seeds of *Pisum sativum.*	69
(3-5)	Specificity of asparaginase purified from *P. sativum* against different substrates	80
(3-6)	Cytotoxicity effect of different concentrations of purified asparaginase from *Pisum sativum* seeds on L20B tumor cell line after incubation for 48 hours measured at 450nm.	85
(3-7)	Cytotoxicity effect of different concentrations of purified asparaginase from *Pisum sativum* seeds on L20B tumor cell line after incubation for 48	86

	hours measured at 492nm.	

List of Figures

No. of Item	Subject	No. of page
(1-1)	Plant parts and flower of *Pisum sativum*	4
(1-2)	Catalysis of L-asparagine hydrolysis by asparaginase	7
)1-3(	Asparaginase families	8
)1-4(	Teriary structure of plant asparaginase	10
)1-5(	Proposed general mechanism of asparaginase reaction	12
)1-6(	Mechanism of action of asparaginase	22
)2-1(	Standard curve of ammonium sulfate for determination of ammonia concentration	38
(2-2)	Standard curve of bovine serum albumin for determination of Protein concentration	40
(3-1)	*Pisum sativum* plant collected for the production of asparaginase	50
(3-2)	Effects of substrate concentration on the activity of asparaginase extracted from seeds of *Pisum sativum*	55
(3-3)	Effect of time of reaction on asparaginase activity extracted from seeds of *Pisum sativum*	56
(3-4)	Effect of the buffer pH on the activity of asparaginase activity extracted from seeds of *Pisum sativum*	58
(3-5)	Effect of temperature on the activity of asparaginase extracted from seeds of *Pisum*	59

	sativum	
(3-6)	Effect of buffer solution on the activity of asparaginase extracted from seeds of *Pisum sativum*	61
(3-7)	Effect of enzyme:substrate ratio on the activity of asparaginase extracted from seeds of *Pisum sativum*.	62
(3-8)	Ion exchange chromatography for purification of asparaginase produced from *Pisum sativum* seeds using DEAE-Cellulose column (2×23 cm) with a flow rate of 20 ml/hour	66
(3-9)	Gel filtration chromatography for purification of asparaginase extracted from *Pisum sativum* seeds using Sephadex G-200 column (1.6×43 cm) equilibrated with potassium phosphate buffer pH8.0,fraction volume was 5ml at flow rate of 20ml/hour	68
(3-10)	Selectivity curve for determining the molecular weight of purified asparaginase extracted from *Pisum sativum* seeds by gel filtration chromatography using Sephadex G-200 (1.6×43 cm)	71
(3-11)	Effect of pH on activity of purified asparaginase extracted from seeds of *Pisum sativum*	72
(3-12)	Effect of pH on stability of purified asparaginase extracted from seeds of *Pisum sativum*	74
(3-13)	Effect of pH on activity of purified asparaginase extracted from seeds of *Pisum sativum*	75

(3-14)	Effect of temperture on stability of purified asparaginase extracted from seeds of *Pisum sativum*	77
(3-15)	Arrhenius plot for determination the activation energy of asparaginase extracted from *Pisum sativum* seeds	79
(3-16)	Cytotoxicity of purified asparaginase from *P. sativum* against L20B cell line using Neutral Red assay	82
(3-17)	Cytotoxicity effect of purified asparaginase extracted from seeds of *Pisum sativum* on L20B cell line after incubation period for 48 hours at 450nm	84
(3-18)	Cytotoxicity effect of purified asparaginase extracted from seeds of *Pisum sativum* on L20B cell line after incubation period for 48 hours at 492 nm	84
(3-19)	Activity of asparaginase purified from *Pisum sativum* and its inhibitory effect on L20B tumor cell line after incubation for 48 hours	88

List of Abbreviations

Abbreviation	Full name
ALL	Acute lymphoblastic leukemia
BSA	Bovine Serum Albumin
Da	Dalton
KDa	Kelodalton
DEAE-Cellulose	Diethyl aminoethyl cellulose
DEAE-Sepharose	Diethyl aminoethyl sepharose
D.W	Distilled water
E.C.	Enzyme code
ELISA	Enzyme-linked immonosorbent assay
I.R	Inhibition rate
IU	International unit
K_m	Michaelis-Menten constant
lµ	Microliter
molµ	Micromole
M	Molar
Mg	Miligram
mM	Milimolar
Min.	Minutes
Nm	Nanometer
O.D.	Optical density
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonyl floride
RNA	Ribonucleic acid
Rpm	Rotation per minutes
RPMI-medium	Roswell Park Memorial Institute-

	medium
SDS-PAGE	Sodium Dodecyl sulfate Polyacrylamide Gel Electrophoresis
Subpp.	Subspecies
TCA	Trichloroacetic acid
V_{max}	Maximum velocity of reaction
U	Units
USDA	United States Department of Agriculture
UV-VIS spectrophotometer	Ultra violate-Visible spectrophotometer
WHO	World Health Organization
W/V	Weight per volume

1. Introduction and Literature Review

1.1 Introduction

Asparaginase is an enzyme that is broadly distributed among the plants, animals and microorganisms. The most commonly used organisms to produce asparaginase are: *Esherichia coli*, *Erwinia carotovora*, *Thermus thermophilius*, *Proteus vulgaris*, *Serratia marcescens*, *Mycobacterium bovis, Streptomyces griseus*, (Kotizia and Labrou, 2005; Oza, 2009), animal organs such as: liver of guinea pig, Placenta Kidney and intestine of beef and horse and in plant tissue as *Pisum sativum* and *Oryza sativa* (Borek and Jaskoliski, 2001).

Pea (*Pisum sativum*) is a member of Leguminecea family that is the widely grown in the world as a source of protein for human food (Faostat *et al.*, 2008). Pea is reported to have potential antioxidant and antimicrobial effect (Saeed and Tariq, 2005; Amarowicz *et al.*, 2001).

The plant asparaginase has been less studied (Borek and Jaskoliski, 2001). In plants, L-asparagine is the major nitrogen storage and transport compound (Sieciechowicz *et al.*, 1988). In *Pisum sativum* and many other legumes asparaginases liberate from asparagine the ammonia that is necessary for protein synthesis. There are two groups of such proteins, called potassium-dependent and potassium-independent asparaginases. Both enzymes have significant levels of sequence similarity (Lough *et al.*,1992b).

Using amino acid sequence and biochemical property as criteria, enzyme with asparaginase activity can be divided into several families (Borek, 2001). The two largest and well

characterized families include bacterial and plant-type asparaginases. The bacterial-type enzyme have been studied for over 40 years (Michalska and Jaskoliski, 2006).

Asparaginase can be effectively used for the treatment of acute lymphoblastic leukemia and tumor cell. The beneficial role of asparaginase administration is usually attributed to the fact that the tumor cells have a compromised ability to generate L-asparagine endogenously, either due to low expression levels of asparagine synthetase or insufficient amount of its substrates, aspartate or glutamine (Stams *et al.*, 2005). Because of their dependence on exogenous L-asparagine, the cancerous acute lymphoblastic leukemia cells, but not normal cells, can be starved and eliminated by asparaginase treatment which depletes the levels of L-asparagine in circulating pools (Aslanian and Kilberg, 2001).

The asparaginase of *Erwinia carotovora* and *E.coli* have only been produced commercially as a drug in the treatment of acute lymphoblastic leukemia, Their main side effects are pancreatitis, diabetes and coagulation abnormalities (Verma *et al.*, 2007). The discovery of new asparaginase serologically different but having a similar therapeutic effect is highly desired (Moharam *et al.*, 2010). Therefore there is a continuing need to screen newer organisms in order to obtain strains capable of producing new, potential source and high yield of asparaginase (Dhevagi and Poorani, 2006). Hence an attempt has been made to find out novel sources of this enzyme from plants.

According to the importance of asparaginase for therapeutical treatments this study was aimed to:

1• Extraction of asparaginase enzyme from different plant parts of *Pisum sativum.*

2• Determination the optimum conditions of crude asparaginase activity.

3• Purification of enzyme using different chromatographic techniques.

4• Characterization of purified asparaginase.

5• Studying the antitumor activity of purified asparaginase against tumor cell line.

Chapter One

Introduction and Literature Review

1.2 Literature Review

1.2.1 *Pisum sativum*

Pea (*Pisum sativum*) is an important legume grown and consumed extensively worldwide (Sarikamis *et al.*, 2010). Pea is a pod-shaped vegetable, small, round, edible seed that are contained within pods (Gritton, 1980). Pea plant usually glaucous; stem weak, 30-150 cm long; leaves alternate (Duke, 1981) as shown in figure (1-1).

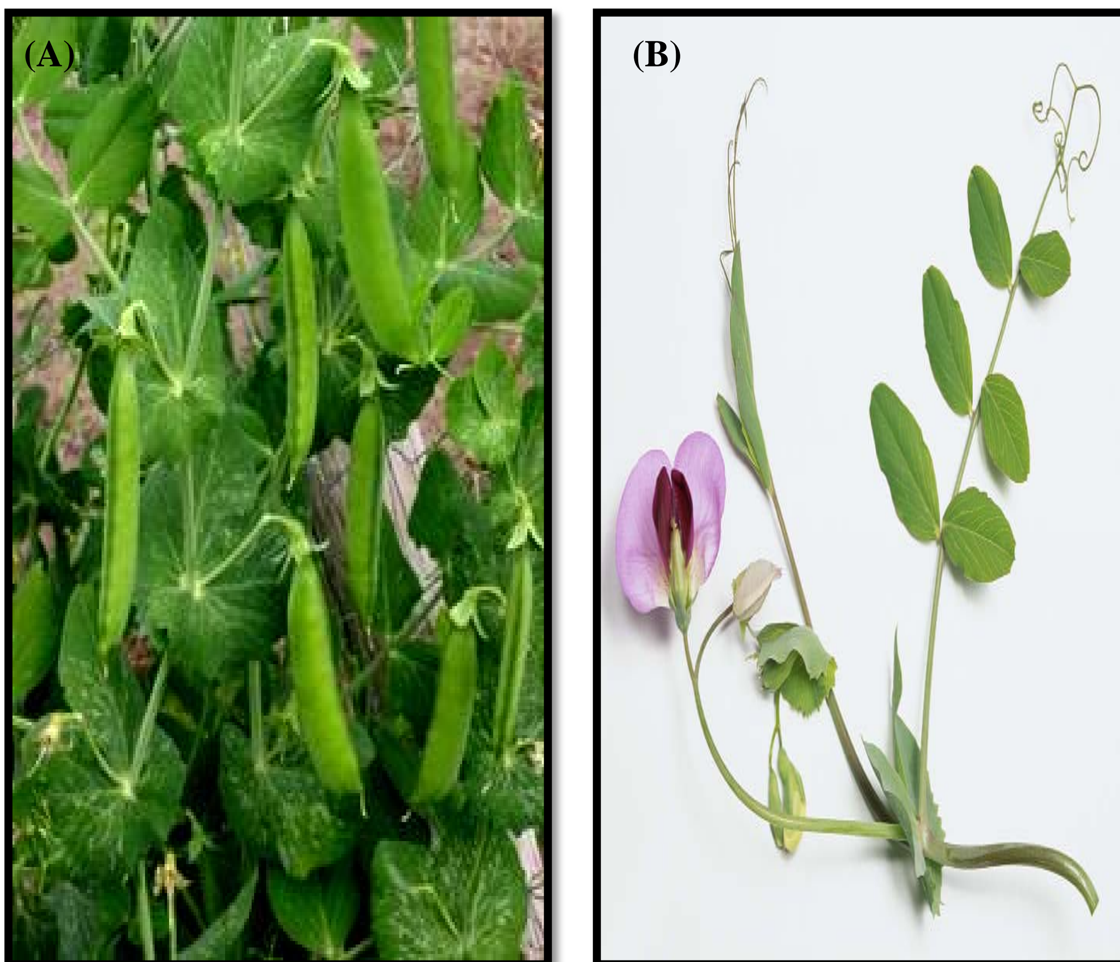


Figure (1-1): Plant parts and flower of *Pisum sativum* (Duke, 1981)
(A) Plant parts (B) leaves with flower

Pea is cultivate either to produce green or garden peas (Hortense) or to produce dry seeds called field peas (Arvense) (Al-Jomaily, 2001). Green pea is eaten cooked as a vegetable, and is marketed fresh, canned, or frozen while ripe dried pea is used whole, split, or made into flour (Davies *et al.,* 1985). In Chinese traditional medicine, the seed of this plant have been described for diuretic, anti-inflammatory, and stomachic purposes (Thiruvikraman *et al.,* 1995).

1.2.2 Classification of *Pisum sativum* (Pea)

Kingdom: *Plantae* – Plants

Subkingdom : *Tracheobionta* – Vascular plants

Superdivision: *Spermatophyta* – Seed plants

Division: *Magnoliophyta* – Flowering plants

Class: *Magnoliopsida* – Dicotyledons

Subclass: *Rosidae*

Order: *Fabales*

Family: *Fabaceae* – Pea family

Genus: *Pisum* L. – pea

Species: *Pisum sativum* L. – garden pea (USDA, 2008)

1.2.3 Nutritional value of *Pisum sativum*

Nutritionally, fresh green peas contain 44 calories per 100 g (Duke, 1981; Hulse, 1994). They have nutritionally favorable composition in respect to macronutrients, low fat, high protein and fiber content (Jokanovic *et al*., 2006). The protein concentration

of peas range from 15-39% (Davies *et al.*, 1985; Bressani and Elias, 1988). The major antioxidants in pea are vitamin C, carotenoid and various phenolic compounds which were present mostly in the cotyledon (Ho *et al.*, 2003; Troszynska *et al.*, 2002).

These unique phytonutrients in green pea also provide us with key antioxidant and anti-inflammatory benefits. Included recently-discovered green pea phytonutrients called saponins (Ohana *et al.*, 1998). Due to their almost exclusive appearance in pea, these phytonutrients actually contain the scientific word for pea (*Pisum*) in their names: pisumsaponins I and II, and pisomosides A and B. (Murakami *et al.*, 2001).The green color is evidence of the chlorophyll present in pea (Hedges and Lister, 2006).

Pea contain numerous enzymes including amine oxidase (Mann ,1995) α -amylase (Eric and Stanley, 1990), protease inhibitor (Liener and Kakade, 1969), L-glutaminase (Rognes, 1980) and asparaginase (Murray and Ireland, 1980; Sodek *et al.*, 1980).

1.2.4 Aspraginase

The enzyme asparaginase (E.C.3.5.l.l) an aminohydrolase catalyses asparagine hydrolysis to yield L-aspartate and ammonia (Borek and Jaskolski, 2001).

The action of asparaginase plays a major role in the cellular nitrogen metabolism of both prokaryotes and eukaryotes (Yossef and Al-Omar, 2008).

1.2.5 Asparaginase hydrolysis

The simple hydrolysis reaction of the side chain amide bond of L-asparagine is catalyzed by a group of amidohydrolases known as asparaginases (Derst *et al.*, 1992) as shown in figure (1-2).

This enzymatic hydrolyses of L-asparagine was first observed by Lang (1904) who detected asparaginase activity in several beef tissues.

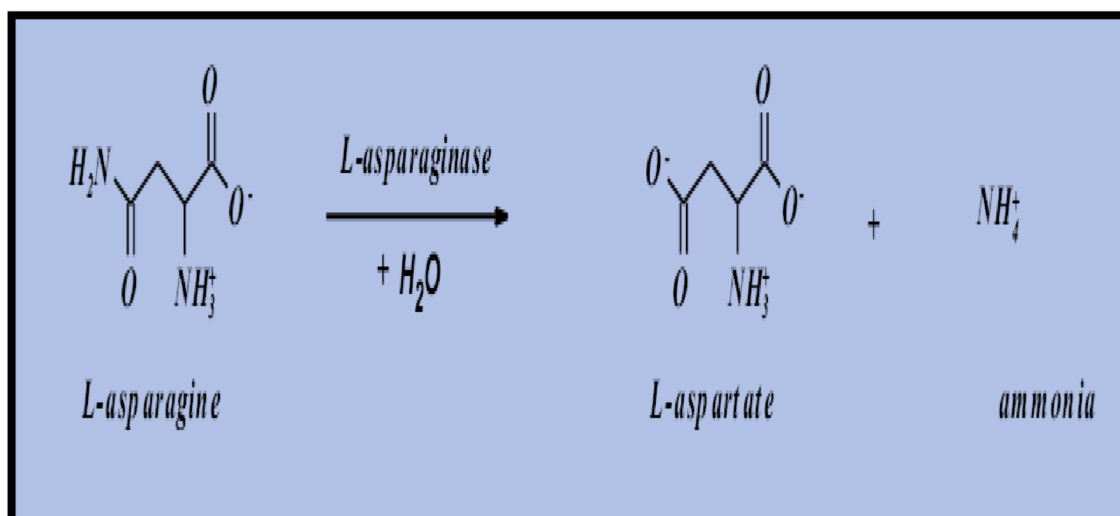


Figure (1-2): Catalysis of L-asparagine hydrolysis by asparaginase (Borek and Jaskolski, 2001).

1.2.6 Asparaginase families

On the dependent of biochemical and crystallographic data, the known asparaginase sequences can be divided into three families as shown in figure (1-3).

The first family corresponds to bacterial-type asparaginase, the second to plant-type asparaginase and the third one to enzymes *Rhizobium etli* asparaginase (Tumbula *et al.*, 2000).

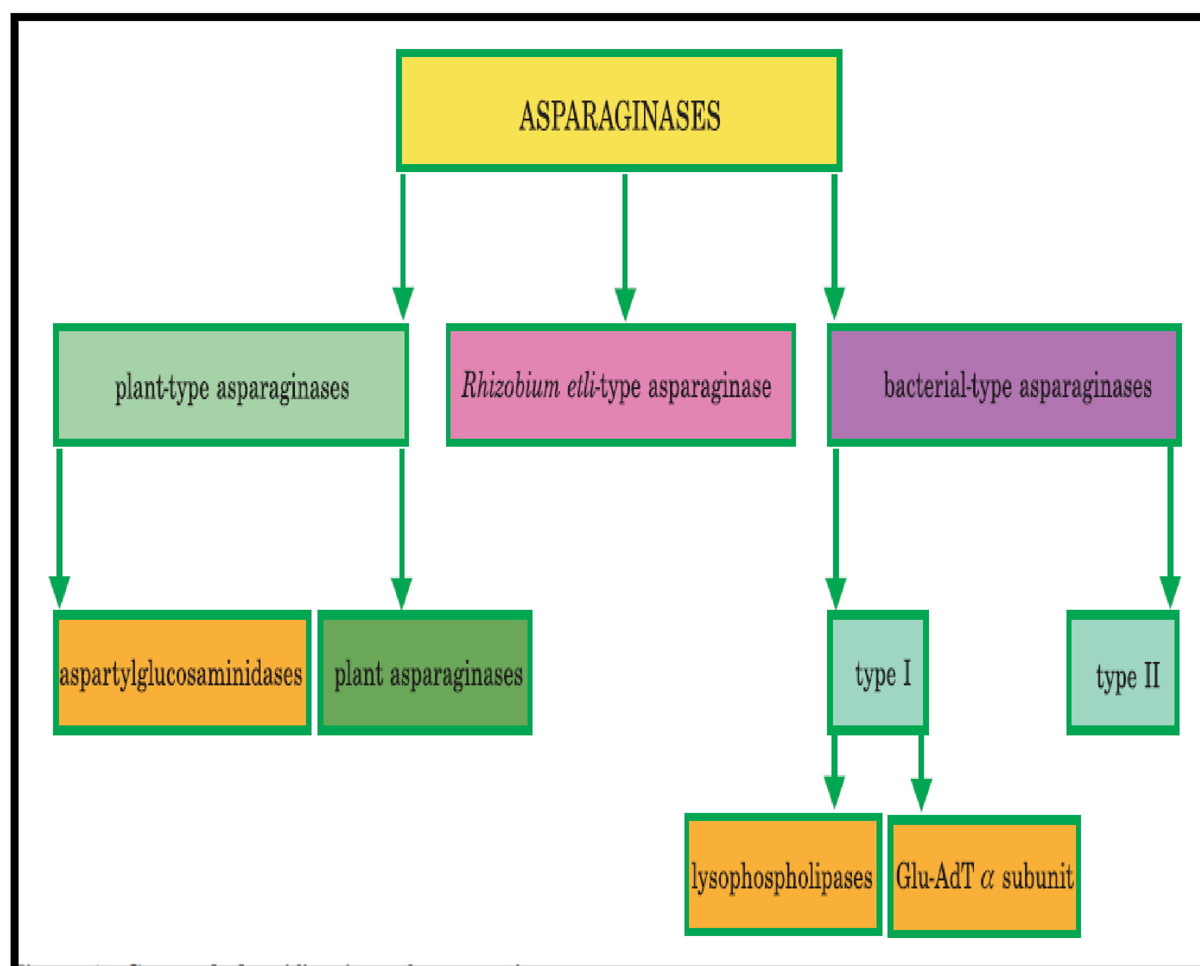


Figure (1-3): Asparaginase families (Borek and Jaskoliski, 2001).

Bacterial-type asparaginases are further divided into subtypes I and II, defined by their intra-/extra-cellular localization, substrate affinity, and oligomeric form(Michalska and Jaskoliski, 2006).

Plant type asparaginases are evolutionarily and structurally distinct from the bacterial-type enzymes. They function as potassium-dependent or potassium-independent asparaginase (Michalska and Jaskoliski, 2006).

1.2.7 Distribution and occurrence of asparaginase

Asparaginase is a widely distributed enzyme and present in plant, animal tissue and microorganisms including bacteria, yeast

and fungi (Siddalingeshwara and Lingappa, 2011). Organisms as reported by many reports produce asparaginase and study their antitumor properties as indicated in table (1-1).

Table (1-1). The main organisms that produce asparaginase with its antitumor properties.

Source	Antitumor activity	Reference
Animal **Guinea pig serum**	 +	Bano and Sivaramakrishnan, (1980)
Plant ***Capscium annum*** ***Pisum sativum*** ***Withania somnifera*** ***Lvcopersicum*** ***Vigna unguiculata*** ***Tamarindus indica***	 + **N.D** + + **N.D** **N.A**	Oza *et al*., (2009)
Bacteria ***E.coli*** ***E. carotovora*** ***Serretia marcescens*** ***Pseudomonas***	 + + + +	Bano and Sivaramakrishnan, (1980)
Yeast ***S. cerevisiae***	 +	Bano and Sivaramakrishnan, (1980)
Actinomycetes ***Streptomyces gulbargensis***	 +	Seema *et al*., (2010)
Fungi ***Aspergillus terreus*** ***Fusarium tricicium***	 + _	Siddalingeshwara and Lingappa, (2011)
Algea ***Chlamydomonas***	 +	Bano and Sivaramakrishnan ,(1980)

N.D. = Not determined **N.A. = Information not available**

\+ **= Have antitumor activity** □ **= Haven't antitumor activity**

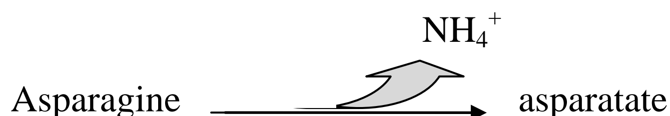
1.2.8 Plant asparaginase

Plant asparaginase belong to the superfamily of N-terminal nucleophile (Ntn) amidohydrolase (Paul, 1982; Michalska *et al*., 2008). The tertiary structure of plant asparaginase was shown in figure (1-4).

Figure (1-4): Tertiary structure of plant asparaginase (Dauter *et al*., 2010).

In plants, L-asparagine is the most abundant metabolite for the storage and transport of nitrogen that is utilized in protein biosynthesis. There are two known routes for L-asparagine metabolism for storage and transport of nitrogen that is utilized in protein synthesis (Borek and Jaskolski, 2001; Borek *et al*., 2004 and Michalska *et al*., 2006).

The first rout, catalysed by asparaginase, involves the hydrolysis of L-asparagine to release ammonia and L-asparatate. The second route, involves the transamination of L-asparagine (in the presence of an oxo-acid) to form 2-oxosuccinamic acid and appears to be important in green leaves where it may play a role in photorespiration (Atkins *et al*., 1983; Murray *et al*., 1987 and Joy, 1988).



1.2.9 General mechanism of the reaction catalyzed by asparaginase

The mechanism of asparaginase has been compared to that of classic serine proteases, whose activity depends on a set of amino-acid residues, typically Ser-His-Asp, known as the "catalytic triad" (Carter and Wells, 1988). This set includes a nucleophilic residue (Ser), a general base (His), and an additional, acidic, residue (Asp), all connected by a chain of hydrogen bonds (Dhavala, 2010).

The reaction consists of two steps as shown in (figure 1-5). In the first step, the enzyme's nucleophile, activated *via* a strong O-H…B hydrogen bond to an adjacent basic residue, attacks the C atom of the amide substrate, leading through a tetrahedral transition state to an acyl-enzyme intermediate product. The negative charge that develops on the O atom of the amide group in the transition state is stabilized by interactions with adjacent hydrogen- bond donors (Dhavala, 2010).

The constellation of those donors (which typically are main-chain N–H groups) is known as the "oxyanion hole".

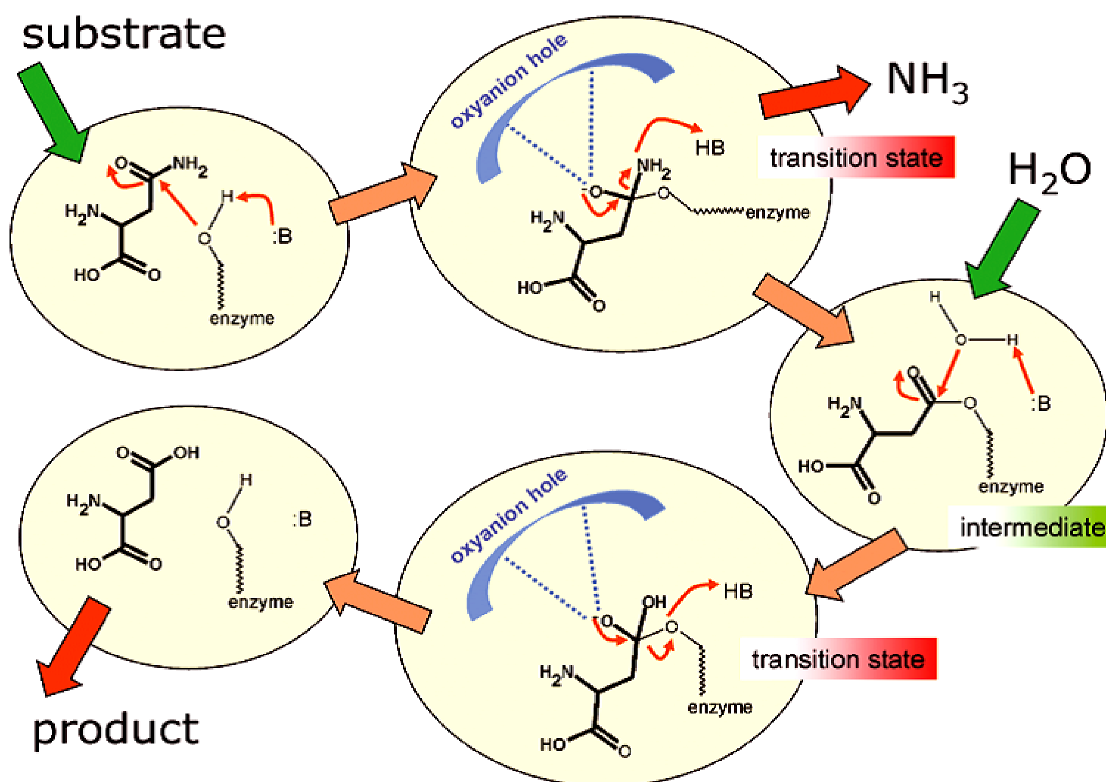


Figure (1-5): Proposed general mechanism of asparaginase reaction (Dhavala, 2010).

The second step of the reaction is similar, but now the attack on the ester C atom is launched by an activated water nucleophile. This useful simple picture is not without doubts, however. One of them concerns the identification of a suitable general base for the activation of the nucleophilic residue.

1.2.10 Molecular structure of plant-type asparaginase

In the literature published in the 1980s, plant enzymes with asparaginase activity were classified as potassium-dependent or potassium-independent form (Sodek *et al.*, 1980), The last reference also reported that K-dependent form found in *Pisum sativum* and other Legume species with higher activity. Both forms were used for the formation of seeds when protein are

synthesized. It has been stated that asparaginase isolated from the developing seed of *Pisum sativum* was dependent upon the presence of K(+) for activity, although Na(+) and Rb(+) may substitute to a lesser extent. Maximum activity was obtained at K(+) concentrations above 20 millimolar. Potassium ions protected the enzyme against heat denaturation. Potassium-dependent asparaginase activity was also detected in the developing seeds of *Vicia faba, Phaseolus multiflorus, Zea mays, Hordeum vulgare*, and two *Lupinus* varieties (Sodek *et al*., 1980).

Molecular structure of plant asparaginases took a leap forward when Hejazi *et al.,*(2002) were able to express the *Arabidopsis thaliana* gene in *E. coli.* Borek *et al*., (2004) expressed a gene encoding the *L. luteus* K-independent asparaginase in *E. coli*.

The availability of the complete sequence of the *A. thaliana* genome allowed Bruneau *et al*., (2006) to isolate a second gene encoding an asparaginase enzyme that was dependent on K^+ for full activity. The two genes showed largely overlapping patterns of developmental expression, but in all the tissues examined, the transcript levels of the K-dependent enzyme were lower than those of the K-independent enzyme (Schmid *et al*., 2005).

So There is an important role for transcriptional control of an asparaginase gene in regulating asparaginase levels in N-sink tissues (Murray and Micheal, 1994).

The current efforts focus on investigation the molecular and structural properties of asparaginase enzyme using molecular and homology modeling of plant asparaginase using bioinformatics tools, from an entirely new sources of asparaginase (Oza *et al*., 2010; Oza *et al*., 2011).

Oza *et al*., (2011) indicated that a significant homology was found with *A. thaliana* and human Taspase1 and some negligible similarity and homology with *E coli*.

1.2.11 Subcellular Localization of asparaginase

Detection the enzyme position is one of the important task that should take in consideration in relation to asparaginase specially for enzyme production and extraction. Many studies has been reported that asparaginase could be extra or intra-cellularly secretion according to the nature of microorganism (Cedar and Schwartz, 1976).

This enzyme in some bacteria accumulate mainly in periplasmic space (Moharam *et al*., 2010). Modern studies proved that asparaginase from *E.coli* is one of the enzymes that only secreted intra-cellularly during normal growth of the bacterial cell (Jerlstrom *et al*., 1989; Jennings *et al*.,1995).

On the other hand, Arima *et al*., (1972) reported that asparaginase secretion from some types of fungi, bacteria and yeast is outside of the cell. In relation to higher organism, very little studies has been reported.

Rogez *et al*., (1975) mentioned that both asparaginase form I and II from Guinea pig liver are secreted in the cytoplasm and mitochondria respectively. while Ireland and Joy, (1983) isolate the enzyme from the protoplast of *Pisum sativum* leaves.

1.2.12 Application of asparaginase

Microbial enzymes, such as asparaginase, were preformed to plant or animal sources due to their economic production, consistency and ease of process modification (Sabu *et al.,* 2005).

In general, plant enzymes are relatively more stable at wide range of pH and temperature than corresponding enzyme derived from microorganisms and animals.

In recent years, asparaginase has attracted much attention in both pharmaceutical and food industrial applications. In food industry, it was used to determine and eliminate acrylamide, from bread using gene technology by degrading L-asparagine, the precursor of acrylamide, prior to backing (Taeymans *et al*., 2005). Another important application of asparaginase is in biosensors when the Indian team of Neelam Verma use asparaginase for development of a novel diagnostic biosenser for the detection of L-asparagine in Leukemia cells (Verma *et al*., 2007).

Recombinant, immobilized and modified asparaginase has been produced from microbial sources with increased activity than wild type sources (O'Driscoll *et al*., 1975; Abshire *et al*., 2000 and Wang *et al*., 2001).

Asparaginase is a therapeutically important protein used in combination with other drugs in the treatment of acute lymphocytic leukemia (mainly in children), Hodgkin's disease, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma, reticlesarcoma and melanosarcoma (Tabandeh and Aminlari, 2009; Sunitha *et al*., 2010).

1.2.13 Optimum condition for asparaginase production

Optimum conditions for activity of any enzyme *in vitro* are not necessarily optimum for the same enzyme *in vivo* (Prakash *et al*., 2009). So the components and requirements of reaction were determined. Factor influencing the detection of asparaginase enzyme like substrate concentration, reaction time, pH,

temperature, buffer type and enzyme:substrate ratio and were optimized by a single factor of varying the parameters one at a time (Sivakumar *et al*., 2006).

1.2.13.1 Substrate concentration

Increasing the substrate concentration increases the rate of reaction (enzyme activity). However, enzyme saturation limits reaction rates. An enzyme is saturated when the active sites of all the molecules are occupied most of the time. It has been shown experimentally that if the amount of the enzyme is kept constant and the substrate concentration is then gradually increased, the reaction velocity will increase until it reaches a maximum. At the saturation point, increases in substrate concentration will not increase the velocity, the reaction will not speed up, no matter how much additional substrate is added (Segal, 1975).

1.2.13.2 Reaction time

It has been suggested that optimization of reaction time is needed to determine the highest amount of enzyme production (Razak *et al*., 1994).

1.2.13.3 pH

As recorded by Bello *et al*., (2011) any increase or decrease of pH from the ranges would cause decrease in the activity of the enzyme, and that could be a good way of controlling undesirable change caused by foods. It has been stated that ionizable groups of the protein structure of enzymes are affected by the pH of the food medium. Changes in pH may not only affect the shape of an enzyme but it may also change the shape or charge properties of

the substrate so that either the substrate cannot bind to the active site or it cannot undergo catalysis. Any change in this pH significantly affects the enzyme activity and/or the rate of reaction (Tipton and Dixon, 1983).

1.2.13.4 Temperature

Like most chemical reactions, the rate of an enzyme-catalyzed reaction increases as the temperature is raised. A ten degree Celsius rise in temperature will increase the activity of most enzymes by 50 to 100%. Variations in reaction temperature as small as 1 or 2 degrees may introduce changes of 10 to 20% in the results. Morimura and Sonada, (1994) have reported that the rate of enzyme catalyzed reactions increases with temperature up to a certain limit. Above a certain temperature enzyme activity decreases because of enzyme denaturation.

1.2.13.5 Buffer

Composition can have significant effects on enzymatic activities. Some buffer components can also affect compound inhibitory activities. Various components in the buffer can be used as factors to modify in a statistical optimization experiment. For best results, published literature information should be used in selecting these factors (Daniel *et al.*, 2010).

1.2.13.6 Enzyme: substrate ratio

In order to study the effect of increasing the enzyme concentration upon the reaction rate, the substrate must be present in an excess amount; i.e., the reaction must be independent of the substrate concentration. Any change in the amount of product

formed over a specified period of time will be dependent upon the level of enzyme present (Daniel *et al*., 2010).

1.2.14 Purification of asparaginase

Protein separation (or extraction) is used to purify a particular protein from some biological (cellular) material or bioproduct since proteins are only synthesized by living systems. The objective to separate the protein of interest from all other non-protein materials and undesired proteins. Enzyme separation is influenced on the interested enzyme activity and structure preparing the purified enzyme to be suitable for medicinal and clinical uses (Clive, 2002).

Asparaginase of *Pisum sativum* was purified approximately 1328-fold with a yield of 1% by anion exchanger with LH-20 and DEAE-Sephacel followed by gel filtration chromatography with Sepharose and Sephacryle S-200. The molecular weight of asparaginase was 69000 Dalton by Sephacryl S-200 chromatography and also by mobility on native SDS-PAGE relative to BSA (Chagaz and Sodek, 2001). Oza *et al.,* (2009) was able to purify asparaginase from *Withania somnifera* by using several purification steps, the purified enzyme gave high specific activity reached to 1540 U/mg, the purification folds arrived to 13.14 and enzyme recovery was 47%.

In other study, obtained purified asparaginase from *Lupinus arboreus* by more than one step includes precipitation by $MnCl_2$ 60% saturation and by $(NH_4)_2SO_4$ precipitation 30-70% then gel filtration with Sephacryl S-200 followed by ion exchange chromatography using DEAE-Sepharose and finally by SDS

polyacrylamide gel electrophoresis (SDS-PAGE) to obtain 205-fold (Chang and Farnden, 1981).

1.2.15 Characterization of asparaginase

1.2.15.1Molecular weight determination of asparaginase

Asparaginases molecular weight differ according to their source, for example asparaginase purified from *Vigna unguiculata* with a molecular weight of 70000 Dalton using Sephacryl S-200 chromatography determined by Mohammad Ali, (2009). While asparaginases purified from *Capsicum annum* have a molecular weight of 120,000 Dalton (Bano and Sivaramakrishnan., 1980).

On another hand the molecular weight of asparaginase purified from *Withania somnifera* was detected by PAGE (polyacrylamide-gel electrophoresis) revealed dense bands along the gel with purified enzyme (Majeed, 2011) and stated to have 72000 Dalton by gel chromatography technique (Oza *et al.*, 2009).

1.2.15.2 Optimum pH for asparaginase activity and stability

Since enzymes are proteins, they are very sensitive to change in pH. Each enzyme has its own optimum range for pH where it will be most active as a result of the effect of pH on a combination of factors (Clive, 2002):

(1) Binding of enzyme to substrate.

(2) Catalytic activity of the enzyme.

(3) Ionization of the substrate.

(4)Variation of protein structure.

The detection of optimum pH for enzyme stability consider necessary task to provide suitable environment for enzyme storage (Hussain, 2005).

Asparaginase purified from *L. arboreus* was found to be most active at pH 8.0, while asparaginase from another species for the same plant (*L. angustifolius*) showed a broad pH activity profile with a maximum of 8.5. The optimum pH of 8.5 for *Withenia sominefera* asparaginase that resembled that of *E.coli* (Majeed, 2011).

1.2.15.3 Optimum temperature for asparaginase activity and stability

Most of the enzymes in nature are characterized by their susceptibility to high temperatures, since it influence the secondary, tertiary and quaternary structure of the enzyme, so lead to denaturation and loss its activity (Whitaker, 1972).

Animal asparaginase from chicken liver showed maximum activity when incubated at 60°C for 20 minutes (EL-Sayed *et al*., 2011a). While plant asparaginase from green *chilie* plant has a temperature optimum of 37°C for 60 minutes. The energy of activation for asparaginase was equal to 11000 calories/mole in *Erwinia aroideae* which has been compared with 6000 calories/mole for *S. cerevisiae* asparaginase (Dunlop *et al*., 1978). In general, other studies have been mentioned that most asparaginases reach maximum activity at 37°C (Heinemann and Howard, 1969; Peterson and Ciegler, 1969). Temperture Coefficient (Q_{10}) value was 1.4 and 1.9 for each of asparaginase I and II purified from *E.coli* respectively (Dunlop *et al*., 1978).

1.2.15.4 Enzyme Specificity

One of the properties of enzymes that makes them so important as a diagnostic and research tools is the specificity they exhibit

relative to the reactions they catalyze. In general, there are four distinct types of specificity: absolute specificity, group specificity, linkage specificity and stereochemical specificity (Daniel *et al*., 2010).

Bruneau *et al*., (2006) showed that potassium dependant asparaginase is strictly specific for L-asparagine. While slight specificity has been showed for potassium-independent enzyme from the *A. theliana* plant.

Asparaginase from *L. arboreus* enzyme hydrolyze only L-asparagine and DL-aspartyl hydroxamte. The same enzyme was inhibited by D-asparagine, L-asparatate, Glutamine, Glutamine analogs and a number of other amino acid (Chang and Farnden, 1981).

In agree with the previous report asparaginase purified from *Tetrahymena pyriformis* was specific for L-asparagine, it doesn't hydrolyze L-glutamine. Its reaction is inhibited competitively by D-aspartic acid and D-asparagine as well as by L-asparagine analogues (Triantafillou *et al*., 1988).

1.2.16 Asparaginase: a promising chemotherapeutic agent

The growth of malignant and normal cell depends on the availability of specific nutrients used in the synthesis of proteins, nucleic acids and lipids, some of the nutrients can be synthesized within the cell, but others are needed to be supplied through the circulating systems (essential amino acids, essential fatty acids, etc.).

Cancer cells exhibit rapid growth and cell division, and therefore have an increased nutritional need than normal cell (Sudarslal, 2000).

L-asparagine is an endogenous amino acid necessary for the function of some neoplastic cells, such as lymphoblasts. In most human cells deficiency of L-asparagine can be compensated by alternative synthesis pathway through which L-asparagine is produced from aspartic acid and glutamine by asparagine synthetase.

Depletion of L-asparagine from plasma by asparaginase results in inhibition of RNA and DNA synthesis with the subsequent cell apoptosis (Piatkowska-Jakubas *et al.*, 2008) as shown in figure (1-6).

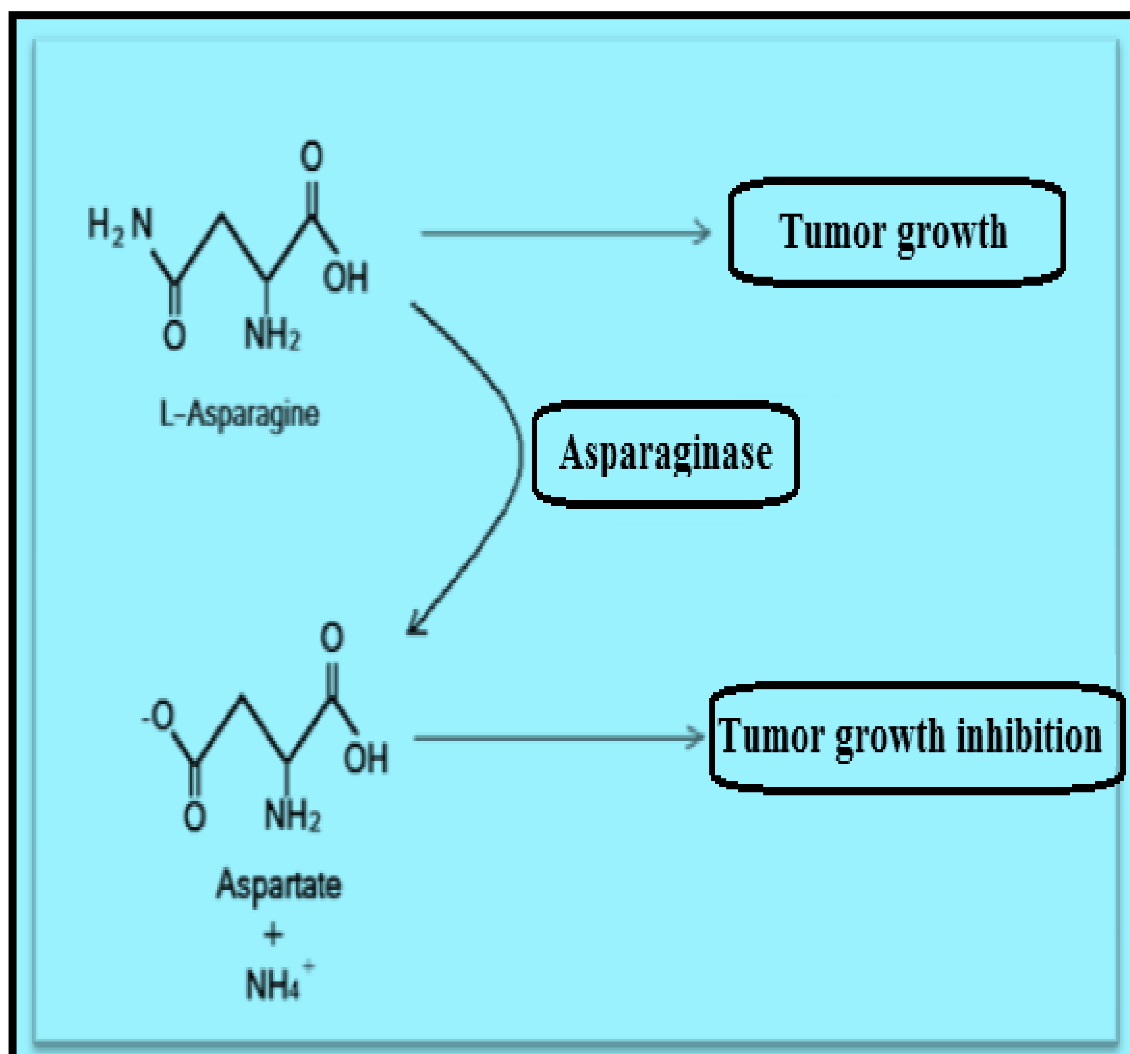


Figure (1-6) Mechanism of action of asparaginase (Narta *et al.*, 2007).

Since tumor cells need asparagines for growing and functioning whereas normal cells can produce enough of this amino acid to meet their requirements with the help of asparagine synthestase (Broome, 1963). Tumor cells are destroyed by L-asparaginase without significant damage to normal cells (Hoffman, 1970).

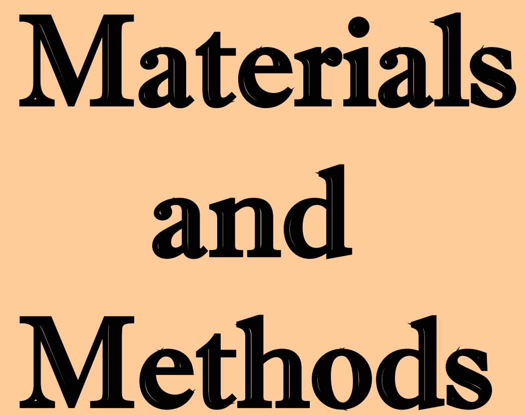
1.2.17 Leukemic treatment with asparaginase

Modern clinical treatments of childhood acute lymphoblastic leukemia (ALL) employ enzyme-based methods for depletion of blood asparagine in combination with standard chemotherapeutic agents (Richards and Kilberg, 2006). In human, acute lymphoblastic leukemia cell lines have been markedly inhibited by asparaginase as the cell cycle arrest in G1 phases (Ueno *et al.*, 1997).

E.coli asparaginase has found to phosphorylate endogenous polypeptides in immune cells. Products of asparaginase specially NH_4^+ ion diffuse into the cytosol and modify the pH, which activates signal transduction pathways associated with phosphorylation of substrates (Mercado and Arenas, 1999). Kelo *et al.*, (2002) have reported that asparaginase action on peptides and their effect on metabolism in the human body (Chakrabarti and Schuster, 1997). The logic of asparaginase therapy is that by delivering asparaginase to the bloodstream plasma asparagines which is quickly depleted causing a rapid efflux of cellular L-asparagine, also destroyed, and thus, the cells of the entire body are depleted of asparagines (Hersh, 1971). Most cells express sufficient asparagines synthesase to counteract this asparagines starvation and survive (Capizzi and Holcenberg, 1993). But in general, childhood ALL cells express asparagines synthesase at a

low level, and therefore, treatment with asparaginase is extremely effective in blocking growth of this particular form of leukemia (Chabner and Loo, 1996).

Chapter Two



2. Materials and Methods

2.1 Materials

2.1.1 Equipments and Apparatus

The following equipments and apparatus were used in this study:

Equipment	Company /origin
Autoclave	Express /Germany
Vortex	Stuart scientified /UK
Balance (sensitive)	Ohaus /Germany
Centrifuge	Humax 4K /Germany
Cooled centrifuge	Selecta p /Spain
CO_2 Incubator	NuAire Laboratory/ USA
Distillator	Kent /England
Digital Camera	Canon/ China
ELISA reader	Organon Techniqa /Germany
Freezer	Arcelic /Turkey
Fraction collector	Sartorius /Germany
Incubator	BDH /England
Magnetic stirrer with hot plate	Stuart scientified
Micropipette	Humax L/ Germany
Microscope	Olympus /Japan
Microtiter plate	Sigma/ USA
Millipore filter paper unit	Millipore crop /USA
Oven	Gellenkamp /UK
PH meter	Metler-Tolledo /UK
UV. Visible Spectrophotometer	CECIL 1000 series /France
Vacuum pump	Scuco Inc. /England

Water bath	Kotterman /Germany

2.1.2 Chemicals and Biological Materials

The following chemicals and biological materials were used in this study:

Material	Company /Origin
Absolute ethanol	Sigma /USA
Acetic acid	Sigma
Ammonium Sulfate	BDH /England
L-Asparagine	Phramacia fine chemicals /Sweden
L-Asparatic acid	Phramacia fine chemicals
Blue dextran-2000	Phramacia fine chemicals
Bovine Serum Albumin	Sigma
Coomassie blue G-250	Fluka /Switzerland
Diethylaminoethyl Cellulose (DEAE-Cellulose)	Sigma
Dipotassium hydrogen phosphate	Riedel-Dehaeny /Germany
Ethelenediaminetetraacetic acid (EDTA)	Fluka
Ethylenediaminetetraacetic acid sodium salt (Na_2-EDTA)	Sigma
Fetal bovine serum	Sigma
Glycerol	Sigma
L-glutamic acid	Phramacia fine chemicals
L-glutamine	Phramacia fine chemicals
Hydrochloric acid (HCl)	Sigma

Penicillin	Sigma
Phenylmethylsulfonylfluoride (PMSF)	Sigma
Phosphoric acid	Sigma
Potassium dihydrogen phosphate	Riedel-Dehaeny
Nesseler reagent	Phramacia fine chemicals
Neutral red dye	Sigma
Sephadex G-200	LKB /Sweden
Sodium chloride	BDH
Sodium hydroxide	BDH
Streptomycin	Sigma
Trichloroacetic acid	Sigma-Aldrich/ USA
Tris-HCl	Sigma
Trypsin	Sigma and PAA/USA
Urease	Riedel-Dehaeny
Versin	Sigma

2.1.3 Solutions, buffers and indicators :

2.1.3.1 Nessler's Reagent

It was used as supplied from manufacturing company (Pharmacia fine chemicals).

2.1.3.2 Potassium phosphate buffer (0.05M)(Good and Izawa, 1972)

It was consist of two solutions :

Solution (A) : potassium dihydrogen phosphate (0.05 M)

It was prepared by dissolving 1.70 g of KH_2PO_4 in 100 ml of distilled water.

Solution (B) : dipotassium hydrogen phosphate (0.05 M)

It was prepared by dissolving 0.85 g of K_2HPO_4 in 100 ml distilled water.

A volume of 5.3 ml of solution (A) was added to 94.7 ml of solution (B), then diluted with distilled water to a total of 200 ml, pH 8.0.

2.1.3.3 L-asparagine solution (200mM, pH=8.0)

It was prepared by dissolving 2.64 g of L-asparagine in 100 ml of potassium phosphate buffer prepared in (2.1.3.2), mixed thoroughly and sterilized by autoclaving and stored at 4°C until use.

2.1.3.4 Ammonium sulfate stock solution (0.64mg/ml)(Imada *et al*., 1973)

It was prepared by dissolving 64 mg of ammonium sulfate in 100 ml of distilled water.

2.1.3.5 Trichloroacetic acid solution (1.5 M)

It was prepared as supplied by Sigma-Aldrich company by dissolving 24.5 g of trichloroacetic acid in 100 ml of distilled water, mixed thoroughly until it was completely dissolved.

2.1.3.6 Bovine Serum Albumin stock solution (100 mg/ml) (Bradford, 1976)

It was prepared by dissolving 1g of BSA in 10 ml of distilled water and stored at 4°C until use.

2.1.3.7 Coomassie Brilliant blue G-250 (100 mg/ml) (Bradford, 1976)

It was prepared by dissolving 100 mg of Coomassie blue G-250 in 50 ml of 95% ethanol, then 100 ml of 85% phosphoric acid was added and the volume was completed to one liter with distilled water. The mixture was then filtrated using Whattman filter paper No.1 and kept in a dark bottle at 4 °C.

2.1.3.8 Sodium Hydroxide solution (1N) (Nikolskij, 1964)

It was prepared by dissolving 40 g of NaOH in appropriate volume of distilled water, then the volume was completed to 1000 ml.

2.1.3.9 Ethylenediaminetetraacetic acid EDTA (1mM) (Nikolskij, 1964)

It was prepared by dissolving 0.14 g of EDTA in 50 ml potassium phosphate buffer solution prepared in (2.1.3.2), pH was adjusted to 8.0, then the volume was completed to 100 ml with the same buffer.

2.1.3.10 Glycerol (10%)

Glycerol solution was prepared by adding 10 ml of glycerol in 80 ml of distilled water, then the volume was completed to 100 ml in a volumetric flask.

2.1.3.11 Sodium chloride solution (0.05 M) (Nikolskij, 1964)

This solution was prepared by dissolving 0.29 g of NaCl in 50 ml of potassium phosphate buffer prepared in (2.1.3.2), then the volume was completed to 100 ml with the same buffer.

2.1.3.12 Phenylmethylsulfonylfluoride (1 mM)

This solution was prepared as supplied by Sigma company by dissolving 0.087 g of PMSF in 100 ml of absolute ethanol .

2.1.3.13 Sodium hydroxide solution (0.25M) (Nikolskij, 1964)

This solution was prepared by dissolving 2.5 g of NaOH in suitable volume of distilled water, then volume was completed to 250 ml with distilled water.

2.1.3.14 Hydrochloric acid solution (0.25M) (Nikolskij, 1964)

This solution was prepared by adding 5.2 ml of 37% HCl to appropriate volume of distilled water, then volume was completed to 250 ml with distilled water.

2.1.3.15 Sodium chloride solution (1.5M) (Nikolskij, 1964)

This solution was prepared by dissolving 2.32 g of NaCl in 80 ml of 0.05 M potassium phosphate buffer solution (pH 8.0), then volume was completed to 100 ml with distilled water.

2.1.3.16 Potassium phosphate buffer (0.1M)(Good and Izawa, 1972)

This buffer solution was prepared to by mixing two solutions:

Solution (A): 0.1 M of KH_2PO_4 (3.40 g per 100 ml distilled water)

Solution (B): 0.1 M of K_2HPO_4 (1.70 g per 100 ml distilled water)

According to Good and Izawa (1972), 5.3 ml of solution (A) was added to 94.7 ml of solution (B), then volume was completed to 200 ml with distilled water at a final pH 8.

2.1.3.17 Blue Dextran solution (4mg/ml)

This solution was prepared by dissolving 40 mg of blue dextran-2000 in 10 ml of 0.1M potassium phosphate buffer solution (pH 8.0) as mention in (2.1.3.16).

2.1.3.18 Phosphate buffered saline PBS (10mM, pH=7.4)

It was prepared as recommended by the manufacturing company (Sigma), then it was sterilized by autoclaving and stored at 4°C.

2.1.3.19 Trypsin solution (2.5%)

This solution was prepared as supplied by PAA company by dissolving 2.5 g of trypsin in 100 ml of PBS.

2.1.3.20 Versine solution (1%)

This solution was prepared by dissolving one gram of ethylenediaminetetraacetic acid sodium salt (Na_2-EDTA) in 100 ml of distilled water.

2.1.3.21 Trypsin-Versine solution

It was prepared by mixing 20 ml of Trypsin, 10 ml of Versine and 370 ml of PBS under aseptic conditions then stored at 4°C until use.

2.1.3.22 Neutral Red solution (0.5%)

This solution was prepared by dissolving 0.5 g of neutral red in 100 ml of acetate buffer solution, then pH was adjusted to 5.2 and

stored at 4° C.

2.1.3.23 Destaining Buffer solution

This solution was prepared by adding 50 ml of PBS solution to 50 ml of absolute ethanol.

2.1.3.24 Trypan Blue solution (0.4%)

This solution was prepared by adding 0.4 ml of trypan blue solution to 99.6 ml of distilled water.

2.1.3.25 Antibiotic solutions

a) Streptomycin stock solution (200 mg/ml)

It was prepared by dissolving 1g of Streptomycin in 5 ml of distilled water.

b) Penicillin stock solution (200 mg/ml)

It was prepared by dissolving 1g of Penicillin in 5 ml of distilled water.

2.1.4 Media

2.1.4.1 RPMI-1640 cell line Growth medium (Freshney, 1994)

This medium was supplied in liquid sterile form and supplemented with L-glutamine, Sodium bicarbonate and Heps buffer, then the following components were added.

Component	Volume
Penicillin	0.5 ml
Streptomycin	0.5 ml
Fetal bovine serum	10%

Then this medium was sterilized by filtration as outline in (2.2.1.3).

2.1.4.2 Maintenance medium (Freshney, 1994)

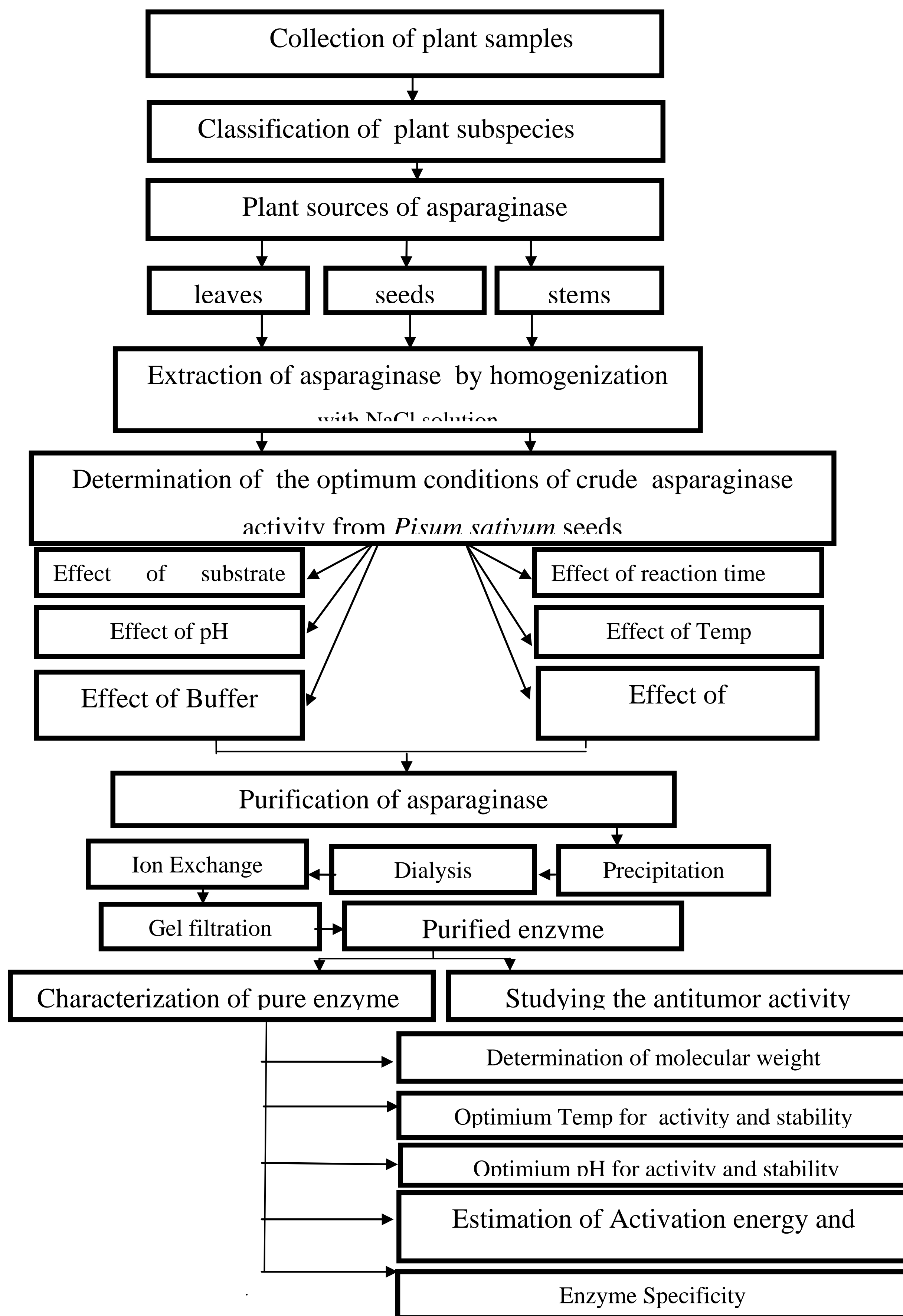
Maintenance medium was consist of the same components of RPMI-1640 cell line growth media except fetal bovine serum which was added in 2% ratio.

2.1.4.3 Cell line

L20B cell line used in this study was kindly provided by the Al-Nahrain Center for Biotechnology Research. This cell line of passage number 18 was genetically engineered mouse cell line expressing the human poliovirus receptor, derived from a human rhabdomyosarcoma (WHO, 2004).

2.2 Methods

Methodology of the research project includes extraction, purification, characterization, and antitumor activity of asparaginase from *Pisum sativum* were illustrated in scheme (2-1).



Scheme (2-1):Methodology of the research project

2.2.1 Method of Sterilization

2.2.1.1 Moist heat sterilization (Autoclaving)

Media, buffers and solutions were sterilized by autoclaving at 121°C (15 Ib/In^2) for 10 minutes.

2.2.1.2 Dry heat sterilization

Glassware were sterilized by dry heat using electric oven at 180 °C for 3 hours.

2.2.1.3 Membrane sterilization (Filtration)

Heat sensitive solutions were sterilized by filtration using Millipore's filter unit (0.22 µM).

2.2.2 Sample collection

Plant parts (leaves, stems and seeds) of *Pisum sativum* were collected during season 2011 from October to March from the field of plant in the University of Baghdad/ College of Agriculture. Healthy looked plant parts were washed three times with distilled water to remove surface dust and other foreign materials, then stored in clean dry container at 4°C until used.

2.2.3 Classification of *Pisum sativum*

Morphological characteristics of *Pisum sativum* were studied for the classification of plant samples according to Horticulture Department/ College of Agriculture/ Baghdad University by Dr. Majid Al-Jomaily.

2.2.4 Extraction of asparaginase

Extraction of asparaginase from plant parts (leaves, stems and seeds) was achieved according to (Chang and Farnden, 1981) by

homogenization, 10 grams of plant parts with three volumes of 0.05 M potassium phosphate buffer, pH 8.0 containing 1.5 M sodium chloride, 1mM PMSF, 1mM EDTA, and 10% (w/v) glycerol, then centrifuged at 10000 rpm for 20 minutes. Supernatant was regarded as crude enzyme.

2.2.5 Enzyme assay

Asparaginase was assayed according to Nesslerization method based on the conversion of L-asparagine to Ammonia and L-asparatate, which has an absorption maximum at 436 nm as it was described by (Ren *et al.,* 2010) as follows:

2.2.5.1 Determination of ammonia concentration

Ammonia concentration was determined according to (Ren *et al.,* 2010) as follows :

In order to determine the ammonia concentration, ammonium sulfate standard solutions was prepared as outlined in (2.2.5.2) according to Imada *et al*., (1973).

2.2.5.2 Ammonium sulfate standard solutions

Stock solution of ammonium sulfate prepared in (2.1.3.4) was diluted for fifty fold of dilution, in order to prepare gradual concentrations of ammonium sulfate (0, 1.6, 3.2, 4.8, 6.4, 8.0, 9.6, 11.2, 12.8 μg/ml) by adding suitable volumes of distilled water to particular volumes of ammonium sulfate (stock solution) as indicated below:

Table (2-1) preparation of ammonium sulfate for standard curve of ammonium sulfate.

Tube No.	Volume of ammonium sulfate stock solution (ml)	Volume of distilled water (ml)	Final concentration of ammonium sulfate(mg/ml)	Absorbance at 436 nm
1	0	8	0.00	**0.00**
2	1	7	1.60	**0.05**
3	2	6	3.20	**0.11**
4	3	5	4.80	**0.16**
5	4	4	6.40	**0.21**
6	5	3	8.00	**0.26**
7	6	2	9.60	**0.32**
8	7	1	11.2	**0.39**
9	8	0	12.8	**0.45**

2.2.5.3 Standard curve of ammonium sulfate

Standard curve of ammonium sulfate was established drown by plotting the relationship between ammonium sulfate concentrations and absorbance at 436 nm as shown in figure (2-1).

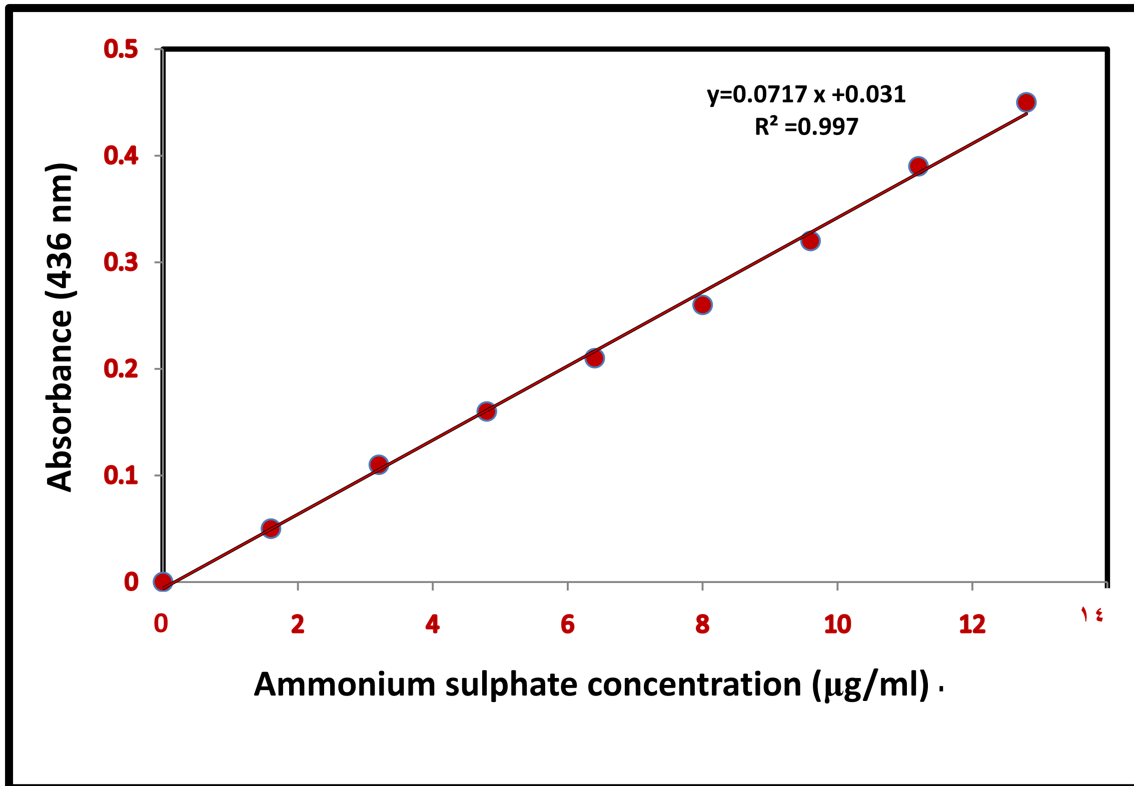


Figure (2-1) Standard curve of ammonium sulfate for determination of ammonia concentration (Imada *et al.*, 1973).

X= Concentration of ammonia, Y= Optical density at 436nm, R^2 = Correlation coefficient (Motalsky and Christopoulos, 2003).

2.2.5.4 Determination of asparaginase activity

Asparaginase activity from plant parts extracts was determined by adding 0.25ml of crude enzyme to 1 ml of 200 mM L-asparagine prepared in (2.1.3.3), then 1ml of potassium phosphate buffer (0.05M, pH 8.0) was added, mixed gently and incubated at 37ºC for 30 minutes. After incubation, 0.5 ml of 1.5M trichloroacetic acid was added to the reaction mixture to stop the reaction, then the mixture was centrifuged at 8000 rpm for 10 minutes, the enzyme activity was measured in the supernatant and the ammonia concentration was determined in clear supernatant by the direct Nesslerization method. Concentration of ammonia was determined for each sample by mixing 3 ml of distilled water

with 0.5 ml of supernatent and 0.5 ml of Nessler reagent. The mixture was shaken well, and incubated at 37ºC for 30 minutes. Then the absorbance was measured at 436 nm. Blank was prepared by mixing 3.5 ml of distilled water with 0.5 ml of Nessler's reagent. One asparaginase unit (IU) is defined as the enzyme amount, Which liberates 1 µmol of ammonia per minute under experimental conditions. Asparaginase activity was calculated according to the following equation (Imada *et al*., 1973)**:**

$$\text{Activity (unit/ml)} = \frac{\text{Concentration of ammonia (µg/ml)}}{\text{Time of}} \quad \text{(Imada } et\ al.\text{, 1973)}$$

$$\text{Specific activity} = \frac{\text{Activity (U/ml)}}{\text{Protien (mg/ml)}}$$

2.2.6 Determination of Protein concentration

Protein concentration was determined according to (Bradford, 1976) using bovine serum albumin (BSA) as a standard protein and as follows :

2.2.6.1 Preparation of Bovine Serum Albumin stock solution

Stock solution of bovine serum albumin (2.1.3.6) was used to prepare gradual concentrations of bovine serum albumin (0, 20, 40

,60, 80, 100 mg/ml) by adding suitable volumes of distilled water to particular volumes of bovine serum albumin as follows :

A volume of 10 µl from each concentration of BSA was added to sterile test tubes, then 250 µl of 1N NaOH and 5ml of coomassie blue G-250 were added to each tube and mixed gently, then it was left to stand at room temperature for 5 minutes followed by reading the absorbancy at 595 nm.

2.2.6.2 Standard curve of bovine serum albumin

Standard curve of bovine serum albumin was drown by plotting the relationship between bovine serum albumin concentrations and absorbance at 595 nm as shown in figure (2-2).

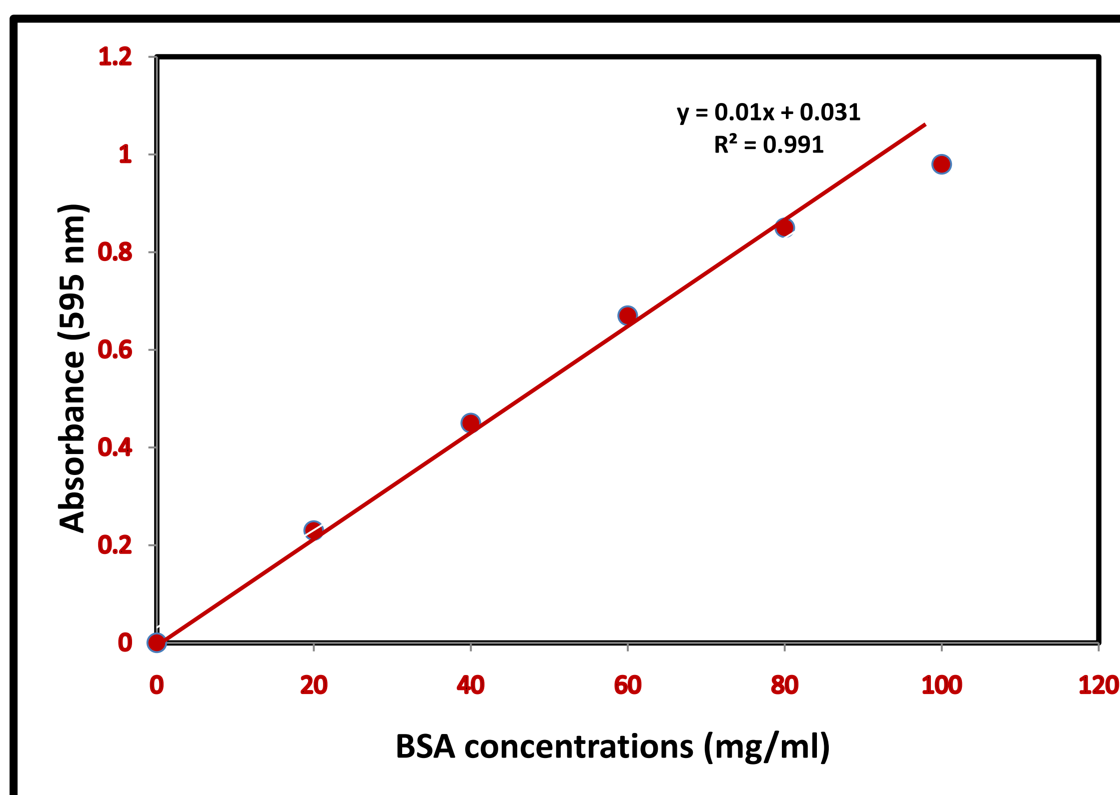


Figure (2-2): Standard curve of bovine serum albumin for determination of Protein concentration (Bradford, 1976).

X= Concentration of protein, Y= Optical density at 595 nm, R^2 = Correlation coefficient (Motalsky and Christopoulos, 2003).

2.2.7 Determination of optimum conditions for crude asparaginase activity

Effect of different factors on crude asparaginase activity were studied according to (Bello *et al*., 2011). These factors include substrate concentration, time of reaction, pH of buffer, reaction temperature, buffer solution and enzyme:substrate ratio.

2.2.7.1 Effect of substrate concentration

Effect of substrate concentration on the activity of crude asparaginase was determined by incubation crude enzyme with different substrate concentrations (10, 50, 100, 150, 200, and 250mM), then asparaginase activity was determined as in (2.2.5.4). Optimum concentration was stated in the next experiments.

2.5.7.2 Effect of reaction time

Effect of reaction time for the enzyme was determined by incubation the reaction mixture for different periods of time (15, 30, 45, 60 and 90 minutes) at 37°C, then enzyme activity was determined as described in (2.2.5.4).

2.2.7.3 Effect of buffer pH

In order to determine the optimal pH for crude asparaginase activity, pH of the reaction mixture was adjusted to different values range (7.5, 8.0 and 8.5). Then enzyme activity was determined as in (2.2.5.4). Optimum pH was stated in the next experiments.

2.2.7.4 Effect of Temperature

Optimal temperature for crude asparaginase activity was determined by incubation the reaction mixture at different temperatures (25, 30, 37 and 40°C). Then enzyme activity was determined as in (2.2.5.4). Optimum temperature for activity was stated in the next experiments.

2.2.7.5 Effect of buffer solution

Effect of the type of the buffer solution for the enzymatic reaction was determined by using two different buffer solutions (0.05M Tris-HCl buffer at pH 8.0, and 0.05 M potassium phosphate buffer at pH 8.0). Enzyme activity was determined as in (2.2.5.4). Optimum buffer was stated in the next experiments.

2.2.7.6 Effect of Enzyme:substrate ratio

Effect of enzyme:substrate ratio on asparaginase activity was determined by using different ratios of enzyme:substrate (1:1, 1:2 and 1:3 Enzyme:Substrate). Then enzyme activity was determined as in (2.2.5.4). Optimum ratio was stated in the next experiments.

2.2.8 Purification of asparaginase

Asparaginase produced by *Pisum sativum* seeds was purified by more than one step using different purification techniques, as mentioned below:

2.2.8.1 Precipitation with ammonium sulfate

The first step of asparaginase purification was achieved by precipitation with ammonium sulfate. Ammonium sulfate was added to the crude enzyme with gradual saturation ratios ranging between 35% and 70% at each saturation ratio, the mixture was

mixed gently on magnetic stirrer at 4°C for more than one hour. Then precipitated proteins were dissolved in a suitable volume of 0.05 M potassium phosphate buffer at pH 8.0. Enzyme activity and protein concentration were determined after each saturation as in (2.2.5.4) and (2.2.6.1) respectively (Segal *et al.*, 1980).

2.2.8.2 Dialysis of enzyme

Crude and ammonium sulfate precipitate enzyme was dialyzed against potassium phosphate buffer (pH=8, 0.05 M) using dialysis tube. Ammonium sulfate precipitate of asparaginase was dialyzed by dissolving the precipitate in 0.05M potassium phosphate buffer, pH 8.0 and dialyzed at 4°C. In the same manner, crude enzyme was dialyzed, for 24 hours with three increments of substitutions. Enzyme activity and protein concentration were determined as in (2.2.5.4) and (2.2.6.1) respectively.

2.2.8.3 Purification by Ion exchange chromatography

2.2.8.3.A DEAE-Cellulose Preparation

DEAE-Cellulose column (2×23cm) was prepared according to Whitaker and Bernard, (1972) by dissolving 20 grams from DEAE-Cellulose resin in 1 liter of distilled water. Then beads were left to settle down left in and were washed several times with distilled water until clear appearance. The suspension was filtered throughout Whattman No.1 Buchner funnel. The resin was then resuspended in 0.25 M sodium hydroxide prepared in (2.1.3.13), filtered again and washed several times with 0.25M hydrochloric acid solution by distilled water before it was equilibrated with potassium phosphate buffer (0.05M, pH8.0).

2.2.8.3.B Sample loading

After column equilibration, concentrated enzyme was transferred and poured gently on to the surface of the column, then washed by potassium phosphate buffer to displace unbinding proteins (wash), Fractions were eluted at a flow rate of 3ml/fraction and the optical density for each fraction was measured at 280 nm. Enzyme activity for each fraction was determined using Nesslerization method described in (2.2.5.4). Other proteins bound to the column was eluted by gradient concentrations of NaCl (0.1-0.5M) dissolved in 0.05 M potassium phosphate buffer solution (pH 8.0). The relationship between eluted fractions, optical density at 280 nm and asparaginase activity were pooled together and kept frozen for the last step of purification by gel filtration chromatography. Fractions represents asparaginase activity were pooled and kept at 4°C for the next step of purification.

2.2.8.4 Gel filtration chromatography

2.2.8.4 A. Preparation of Sephadex G-200 column

Five grams of the gel (Sephadex G-200) were suspended in 1 liter of 0.1M potassium phosphate buffer prepared in (2.1.3.15), then suspension was left in a water bath at 90ºC for 5 hours to ensure the swelling of gel beads with gentle agitation from time to time. Then the gel was degassed, packaged gently in a glass column (1.6x43 cm). The column was equilibrated using the same buffer at a flow rate of 20 ml/hour for 24 hours.

2.2.8.4.B Sample loading

After column preparation, 5ml of the enzyme solution obtained from the elution fractions of ion exchange chromatograph (2.2.8.3.B) was added gently to column surface and eluted using 0.1 M potassium phosphate buffer pH 8.0 with flow rate of 20 ml/hour (5ml for each fraction). Optical density (at 280nm), and enzyme activity (U/ml) were determined in each fraction. Fractions represents asparaginase activity were pooled and kept at 4°C for further studies.

2.2.9 Characterization of purified asparaginase

2.2.9.1 Determination of Molecular Weight

Molecular weight of asparaginase was determined by gel filtration chromatography according to (Andrews, 1970) using sephadex G-200. Four standard proteins listed in table (2-1) were prepared at a concentration of 3 mg/ml.

Table (2-1): Standard proteins used for the determination of asparaginase molecular weight.

Standard protein	Molecular weight (Dalton)
Pepsin	14000
Trypsin	23000
Bovine Serum Albumin	67000
Urease	430000

Void volume of the packed gel was estimated by using Blue dextran 2000. A volume of 3ml of the enzyme sample and standard proteins were added individually to the top surface of

the gel, and fractions were collected at flow rate of (20 ml/hour). And the optical density for each fraction was measured at 280 nm. Then selectivity curve was plotted according to the relationship between log molecular weight of each standard protein and the ratio of Ve/Vo, then asparaginase molecular weight was calculated from the selectivity curve.

2.2.9.2 Determination of optimal pH for asparaginase activity

This can be achieved by using buffer solutions prepared at different pH (4.0-10.5); potassium phosphate, sodium acetate and and NaOH glycine solutions were used to obtain the required value, which had been distributed evenly into aliquots. 0.5 ml of 200mM L-asparagine was added to 0.5ml of buffer solutions at stated pH. Test tubes were incubated in a water bath at 37°C for 10 minutes. Then an enzyme solution was added to the reaction solution and incubated for 30 minutes, then reaction was stopped; the activity of asparaginase was assayed and plotted against optimal pH for asparaginase activity.

2.2.9.3 Determination of optimal pH for enzyme stability

Purified enzyme was incubated at different pH values ranging between 4 and 10.5 at room temperature for one hour. The activity and remaining activity were then measured after assaying enzyme activity.

2.2.9.4 Determination of optimal temperature for enzyme activity

Asparaginase activity was determined after incubation of the

purified enzyme with its substrate at different temperatures (20, 25, 30, 37, 40, 45, 50, and 55°C). Then asparaginase activity was assayed after each incubated temperature.

2.2.9.5 Determination of Activation Energy (Ea)

Activation energy of the purified enzyme was calculated from the relationship between inverse absolute temperature (1/T(K°)) as X-axis versus logarithms of the Reaction rate constant (Log k_{obs}) by using Arihinus slope as following formula (Segal, 1976):

$$Slope = -Ea/2.3R = \frac{\log k_2 \;\square\; \log k_1}{\quad}$$ Segal,

Tempertature Coefficient (Q_{10}) was calculated by applying the following equation (Segal, 1976).

$$\text{Log } Q_{10} = \frac{10\,Ea}{2.3\,R\,T_2\,T_1}$$ (Segal, 1976)

Where

T : $1/T^{\circ} \times 10^{-3} (k\text{-}1)$

Ea : Activation energy

R : Gas constant =1.987 cal/mol/K

K: reaction rate constant

2.2.9.6 Determine the optimal temperature for enzyme stability

A purified enzyme was incubated in a water bath for different temperatures (20, 25, 30, 37, 40, 45, 50, and 55°C) for one hour, then immediately transferred into an ice bath. Activity was assayed for each treatment, Then activity and remaining activity

(%) of asparaginase was plotted against the temperature (°C).

2.2.9.7 Determination of Enzyme specificity

The specificity of purified asparaginase against different substrates was studied by incubating the purified enzyme with L-asparagine, L-aspartic acid, L-glutamine and L-glutamic acids at 200mM, pH 8.0 at optimum temperature for the activity for 30 minutes. Enzyme activity was then assayed after each treatment.

2.2.10 Antitumor activity of purified aparaginase

An *in vitro* study was conducted to investigate the antitumor activity of purified asparaginase enzyme on tumor cell line (L20B), (Freshney, 1994) as follows:

2.2.10.1 Subculture of L20B tumor cell line

cell suspension of L20B tumor cell line was prepared by treating 25ml of cell culture with 2ml of trypsin-versine solution. after the formation of monolayer surface of cell suspension, 20 ml of growth medium which was supplemented with 10% fetal bovine serum was added to inactivate trypsin activity. After that the viability of the cultured cells were counted by using trypan blue. The viability should be more than 95%, then cell suspension was mixed gently and transferred into a micro titer plate (200 µl/well). Each well must be containing $1x10^5$ cell/well. Plates were then incubated at 37°C until 60-70% confluence of the internal surface area of the well for L20B cell lines (Toolan, 1954; Freshney, 1994).

2.2.10.2 Detection of Cell Growth Inhibition

To detect the growth inhibition of tumor cell line L20B, culture of this cell line was incubated with different concentrations (150, 75, 37.5, 18.75, 9.37, 4.68, 2.34 and 1.17µg/ml) of purified asparaginase in six times duplicate for each treatment. Negative control was achieved by incubating culture of cell line with maintenance medium and serum, then plates were incubated at 37°C in an incubator supplemented with (5%) CO_2 for 48 hours. After elapsing the incubation period, 50 µl of neutral red was added to each treatment (50 µl/well) and reincubated for 2 hours. After incubation, cell culture in each plate was washed three times with PBS then 100 µl of destaining buffer solution was added to each well to remove the excess dye from viable cells. Optical density of each well was measured by using ELISA reader at a transmitting wave length 450 and 492 nm. (Freshney, 1994; Mahony *at al.*, 1989). % Remaining activity, % Cell survival and Inhibition rate was also measured. The inhibitory rate was measured according to (Wang *et al.,* 2003) as follows:

$$\textbf{I.R \%} = \frac{\text{O.D. of control - O.D. of test}}{\text{O.D. of control}} \times 100$$ (Wang *et al.,* 2003)

Remaining activity percentage (R.A %) calculated with the following formula:

$$\textbf{R.A\%} = \frac{\text{O.D. of test well (treated cells)}}{\text{O.D. of control well (cells only)}} \times 100$$ (Henson *et al.*,1989)

Cell survival percentage (C.S.%) calculated with the following formula:

$$\textbf{C.S \%} = \frac{\text{O.D. of test well (treated cells)}}{\text{Average of O.D. control well (cells only)}} \times 100$$ (Freshney ,1994)

Chapter Three

3. Results and Discussion

3.1 Collection and Classification of *Pisum sativum*

Plant samples of *Pisum sativum* were collected during season 2011 from the field in the College of Agriculture/ University of Baghdad, figure (3-1). These samples were classified by Horticulture Department/ Collage of Agriculture by Dr. Majid Al-Jomaily according to their morphological characteristics. Results indicated in table (3-1) showed that these plant samples were classified as *Pisum sativum* subspp. *Jof*. The subspecies was characterized with their high quality, high productivity and wide spectrum distribution in Iraq and whole middle east (Abd Al-Hussein, 2001). According to these facts, *Pisum sativum* subspp. *Jof* was selected for the production of asparaginase.

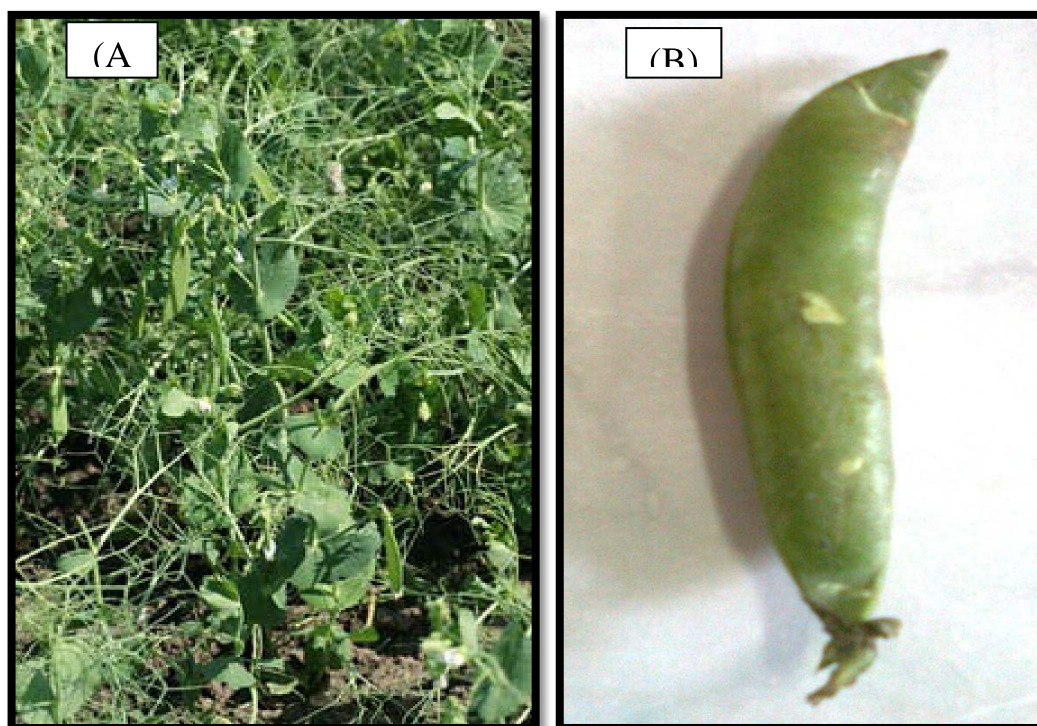


Figure (3-1) : *Pisum sativum* plant collected for the production of asparaginase.

A: Plant parts

B: Pods

Table (3-1): Morphological characteristics of *Pisum sativum* sample.

Part	Characteristic
Growth direction	Random direction
Roots	Branched at one side near the upper surface of the ground
Flowers color	White, flowering began after 77 days
Stems	Intermediate length, weak, slender, long (75 cm height)
Leaves	alternate, pinnate with 1-3 pairs of leaflets and a terminal branched tendril leaflets, 1.5-6 cm long
Pods	pods swollen, curved, 4-15 cm long, 1.5-2.5 cm wide, 2-10 seeded, 26 pods/plant, have the phenomena of Yellowing of the top with the resistance to the fusarium disease
Nodes	The node at which the first flower emerges is characteristic of a given variety, nodes at which the first flower emerges is reported to be 25 in late maturing types under field conditions
Seeds	Seeds are globose, wrinkled, glaucous; 100 seeds can weigh 100 grams

3.2 Detection of asparaginase in plant parts of *Pisum sativum*

Asparaginase activity in plant parts of *P.sativum* was detected by extraction of asparaginase from seeds, leaves, and stems, using the homogenization method with 0.05M of sodium chloride. Results in table (3-2) showed that enzyme activity was detected in the extracts of different plant parts. Maximum activity (605 U/ml) and specific activity (75.6 U/mg) of asparaginase was detected in the extracts of plant seeds, while the enzyme activity and specific activity was less in leaves and stems extracts respectively.

On the other hand, the total activity of crude asparaginase in the extracts of these plan parts were 6050, 5005, and 4880 U in seeds, leaves, and stems respectively. According to these results plant

seeds were used for enzyme production, purification, characterization and applications of asparaginase.

It has been reported that *Pisum sativum* is a source of protease (Guardiola and Sutcliffe,1971), therefore phenylmethylsulfonyl fluoride (PMSF) was used in the buffer of extraction as an inhibitor for serine proteases and alkaline proteases which was active in alkaline pH (Genckal, 2004) and may degrade asparaginase that was active in alkaline pH (pH8-8.5) (Michalska and Jaskolski, 2006).

Further more, using the buffer solution consist of NaCl, EDTA and glycerol for extraction of asparaginase from *Pisum sativum* by active cell lyses that could release high enzyme molecules (Genckal, 2004). In addition, using higher speed of centrifugation (10000rpm) for the homogenization gives high yield of precipitated proteins (Eli and Co, 2001a). Choice of appropriate buffer concentration is essential in the separation methods to avoid non specific ionic strength effects (Ahmed, 2005).

As it has been reported by Michalska *et al*., (2006) who mentioned that the high levels of asparaginase expression have been observed in developing seeds where aparaginase supplies 50-70% of the required nitrogen. In other study it was found that asparaginase was active in plant tissues requiring nitrogen for growth (Lea *et al*., 2007).

Asparaginase produced by ripe fruits of *W. somnifera* had higher enzyme activity in the ripe fruits (0.96 U/ml) than unripe fruits and leaves (0.17 U/ml and 0.18 U/ml respectively) (Majeed, 2011). Other findings mentioned that the asparaginase activity

from the developing seeds of *Lupinus arboreus* and *Lupinus angustifolius* was more than that obtained in the extract of other tissues (Chang and Farnden, 1981).

Table (3-2): Asparaginase activity and specific activity in the extract of plant parts of *Pisum sativum.*

Plant part	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Specific Activity (U/mg)	Total activity (U)
Seeds	14	30.0	6.4	4.6	**14490**
Leaves	14	26.4	4.8	5.5	**369.6**
Stems	14	16.1	4.8	3.0	**225.4**

3.3 Optimum conditions for asparaginase activity

Optimum conditions for asparaginase activity were studied after extraction of the enzyme from plant seeds. These conditions includes the optimum substrate concentration, reaction time, pH of the reaction mixture, temperature of the reaction, type of the buffer solution, and the ratio of enzyme:substrate. Changing in any of these parameters may affect the enzyme activity (Whitaker, 1994). It is possible to improve the enzyme activity assay to have higher activity by increasing substrate concentration, with taking other factors affects enzyme reaction in regards which includes the optimum ionic strength in the reaction (pH), optimum temperature of incubation, optimum salts concentration, time scale and enzyme concentration (Dalaly, 1990).

3.3.1 Effects of substrate concentration

In order to determine the optimum substrate concentration for asparaginase activity, six concentrations (10, 50, 100, 150, 200, and 250mM) of enzyme substrate (asparagine) were used for this purpose.

Results mentioned in figure (3-2) showed that the activity of asparaginase was increased gradually with the increase in L-asparagine concentration. Maximum activity of asparaginase was obtained when the substrate concentration was 200mM, at this concentration, asparaginase activity was 332.7 U/ml. This concentration (200 mM) of L-asparagine was regarded as the optimum for asparaginasee activity, and was used in the next experiments of optimization.

These results were agreed with Al-Noab, (2005) who found that there is a positive relationship between the enzyme activity and substrate concentration, hence the reaction was increased with the increase of substrate concentration when the asparaginase concentration was constant until the maximum rate was achieved (steady state), then the increase of substrate concentration doesn't affects the rate of reaction and doesn’t significantly affects the formation of the product (Silverthorn, 2004).

At the steady state, there is no any enzyme molecules free to act on extra-substrate molecules. In addition, substrate inhibition will sometimes occur when excessive amounts of substrate are present in the reaction mixture (Martinek, 1969).

According to results mentioned in figure (3-2) asparaginase produced by *P. sativum* was reached the steady state when the asparagine concentration was 200mM.

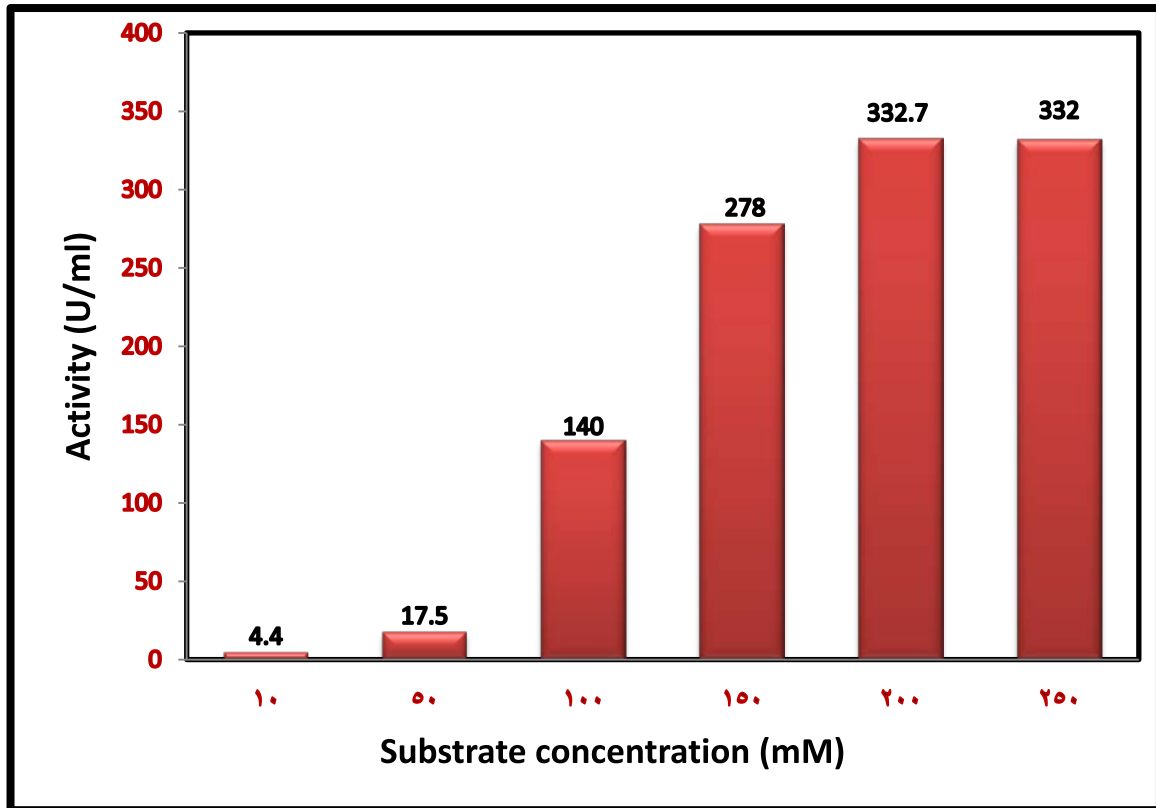


Figure (3-2): Effects of substrate (asparagine) concentration on the activity of asparaginase extracted from seeds of *Pisum sativum* incubated at 37°C for 30 minutes, pH=8.

John *et al*., (1976) reported that consumption of high substrate concentration indicates high activity of asparaginase in the treatment of the late stages of acute lymphoblastic leukemia in the blood of children.

Arnnok *et al*., (2010) reported that the optimum substrate concentration of polyphenol oxidase activity from hot pepper of *Capascium annium L.* was 33mM using catechol as a substrate. While the optimum activity of peroxidase from the same plant was 18mM using guaiacol as a substrate.

3.3.2 Effect of Reaction time

In order to determine the optimum reaction time for asparaginase activity produced by *P. sativum*, different time periods of incubation (15, 30, 45, 60, and 90 minutes) were used.

Results indicated in figure (3-3) showed that the optimum incubation period was 30 minutes, the enzyme activity was 397U/ml. According to these results, it has been concluded that 30 minutes of incubation was enough for asparaginase to bind substrate perfectly in reaction mixture reaching maximum enzyme activity.

Dalaly, (1990) reported that time-scale is an important factor in determining the enzyme activity, and it was preferred to use methods with short time incubation to determine the enzyme activity.

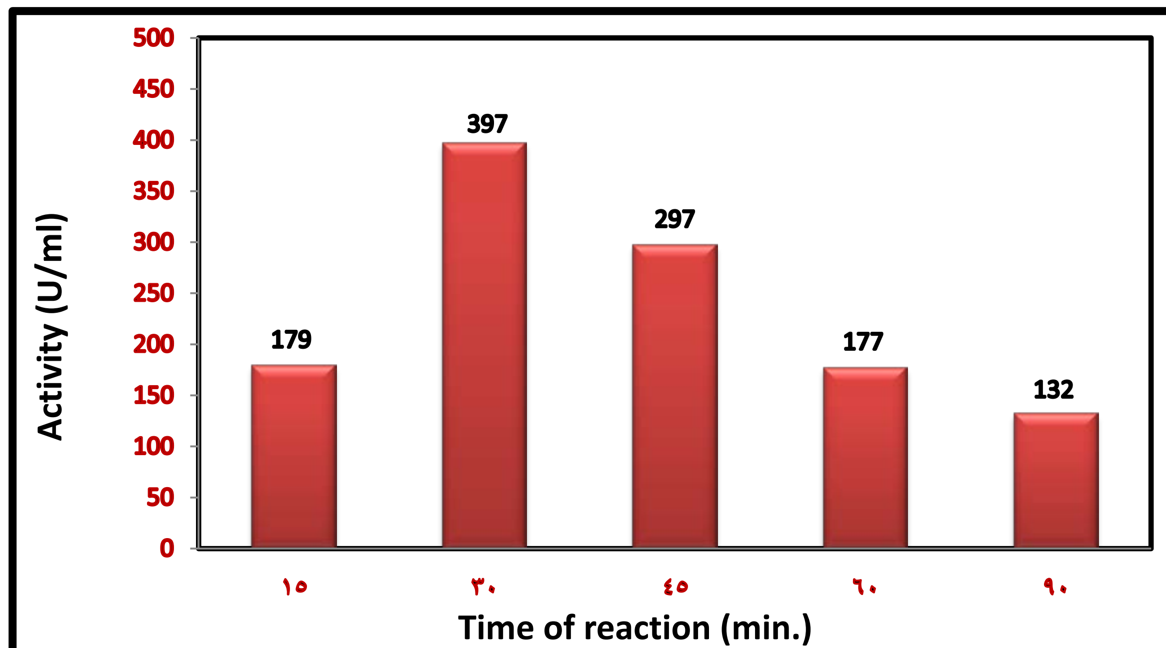


Figure (3-3): Effect of reaction time on asparaginase activity extracted from seeds of *Pisum sativum* using 200 mM aspargine, potassium phosphate buffer pH8 incubated at 37°C.

The effect of incubation time on the crude extract of chitosanase activity from pepper leaves and opuntia peels were studied up to 180 min. It was indicated that chitosanase activity from pepper leaves increased as the reaction time increased up to 180 minutes at 40°C. There is a linear relationship between chitosanase activity and reaction time. In the same manner chitoasanase from opntia peels increased with increasing the reaction time up to 120 minutes at 40°C (El-Sayed *et al*., 2011b).

3.3.3 Effect of buffer pH

Effect of pH on the activity of asparaginase activity produced by *Pisum sativum* was studied. Result illustrated in figure (3-4) showed that maximum asparaginase activity was obtained when pH of the reaction mixture was adjusted to 8.0, at this value, the enzyme activity was 399.5 U/ml. It has been reported that most plant asparaginases have its maximum activity in alkaline pH, and was determined in pH range of 7.5, 8.0 and 8.5 (Oza, 2009). In general most plants show maximum enzyme activity at or near neutral pH (Gawlik-Dziki *et al*., 2007).

It was reported that asparaginase produced from *E.coli* have its maximum activity in alkaline pH that probably due to the balance between L-aspartic acid and L-aspartate. L-aspartic acid in acidic pH has greater affinity to the active site of the enzyme.

Any decrease or increase in hydrogen ions (H^+) concentration causes pH changes in the reaction mixture which may lead to drastic changes in three-dimensional structure of protein, resulting in the enzyme denaturation (Tortora *et al*., 2004). On the other hand, the effect of pH on enzyme activity resulted from its effect on the ionization state of the substrate (Bull and Bushnel, 1976).

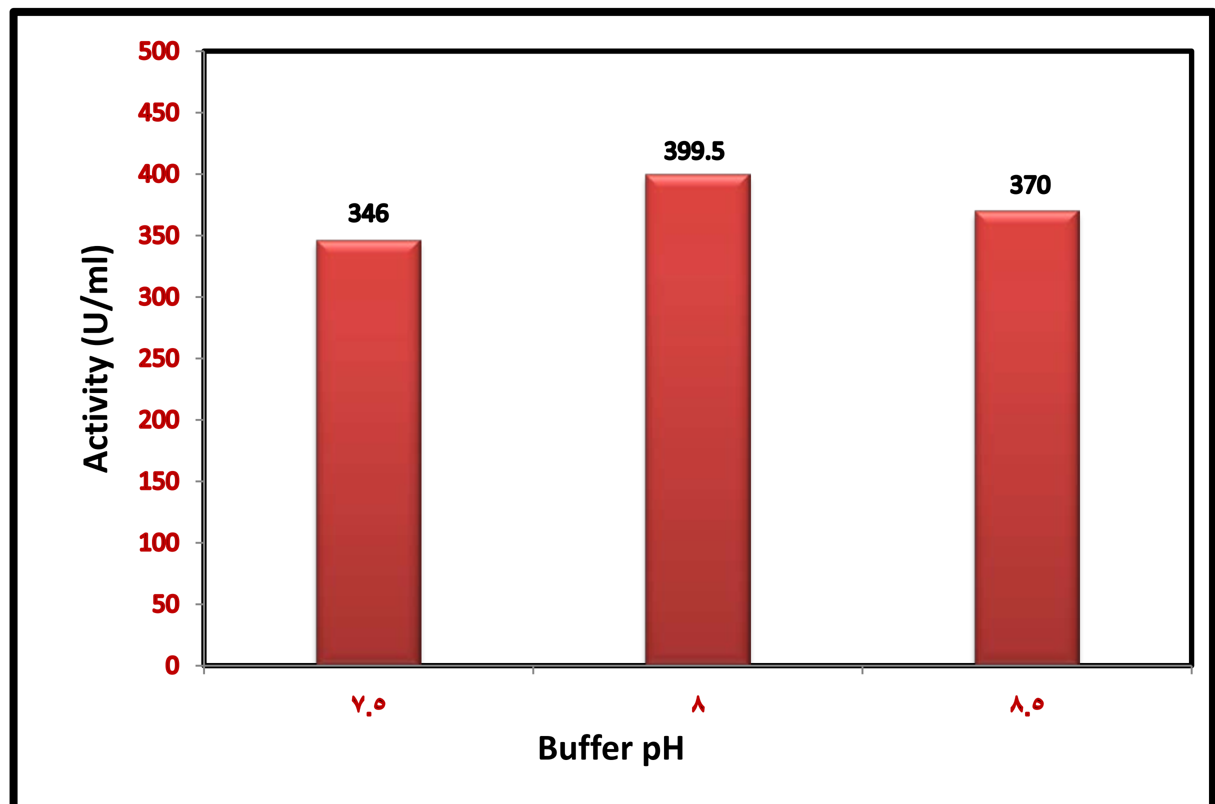


Figure (3-4): Effect of buffer pH on the activity of asparaginase extracted from seeds of *Pisum sativum,* 200mM aspargine, incubation at 37°C for 30 min..

Under such conditions, it becomes a competitive inhibitor. In alkaline pH, the balance is shifted toward the asparatate, which is less affinity to the active site enabling, in this case, there is favorable balance for the connection with the substrate L-asparagine (Miller *et al*., 1993; Lubkowski *et al*., 1994). Da Silva *et al*., (2010) reported that the optimum pH of polyphenol oxidase from *Umbu-caja* (*Spondias* spp.) was 7.0 while Bello *et al*., (2011) found that pH 8.0 was the optimum for polyphenol oxidase from *Irvingia gabonnensis*.

3.3.4 Effect of temperature

In order to determine the optimum reaction temperature for the activity of asparaginase extracted from seeds of *P. sativum*, different temperatures (25, 30, 37, and 40°C) were used for this purpose. It was reported that this range of temperature was the most suitable for enzyme activity (Dalaly, 1990).

Results illustrated in figure (3-5) showed that the maximum activity of asparaginase was obtained when the temperature of the reaction mixture was 37°C. At this temperature, enzyme activity was increased to 488.6 U/ml. In general, it was found that asparaginase from most organisms have its maximum activity at 37°C (Abdel Hameed, 2005). While the increase or decrease in the incubation temperature above or below the optimum temperature cause a decrease in enzyme activity.

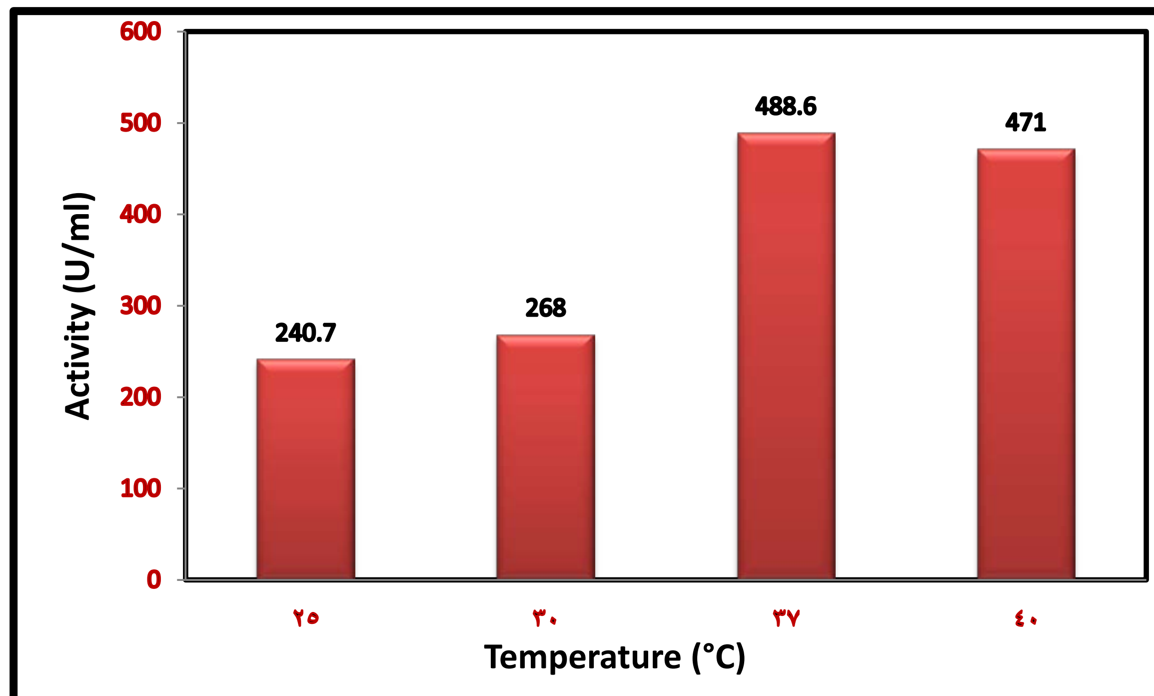


Figure (3-5): Effect of temperature on the activity of asparaginase extracted from seeds of *Pisum sativum*.

These variations in the activity of asparaginase proved that the suitable temperature for asparaginase reaction to have maximum activity was 37°C, while the activity was decreased when the temperature was above optimum or below the temperature.

Dogan and Dogan, (2004) found that the temperature at which polyphenol oxidase from *Thymus logicaulis* showed highest activity was in range of 25-30°C and then decreased at temperature above 40°C. while peroxidase was highly active at 40°C and cost its activity at higher temperature (Alam and Husain, 2007; Saraiva *et al*., 2007 and Belcarz *et al*., 2008).

3.3.5 Effect of buffer solution

In order to determine the optimum buffer solution of the reaction mixture for activity of asparaginase extracted from seeds of *P. sativum*, two types of buffer solutions were used to study their effects on the biocatalysis of the enzyme on its substrate. These buffer solutions are potassium phosphate and Tris-HCl buffer solutions in a concentration of 0.05 M, pH 8.

Results mentioned in figure (3-6) showed that the maximum activity of asparaginase was obtained when potassium phosphate buffer solution was used. Enzyme activity of asparaginase in the crude filtrate was reached 489 U/ml. These results are reasonable with those described by Michalska and Jaskoliski, (2006) who found that potassium phosphate buffer solution was adaptive or inducer for plant asparaginase activity.

It acts as a source of potassium ion for activation of plant asparaginases. Sodek *et al*., (1980) demonstrated that asparaginase

obtained from seeds of *P. sativum*, was dependent on the presence of potassium for enzyme activity.

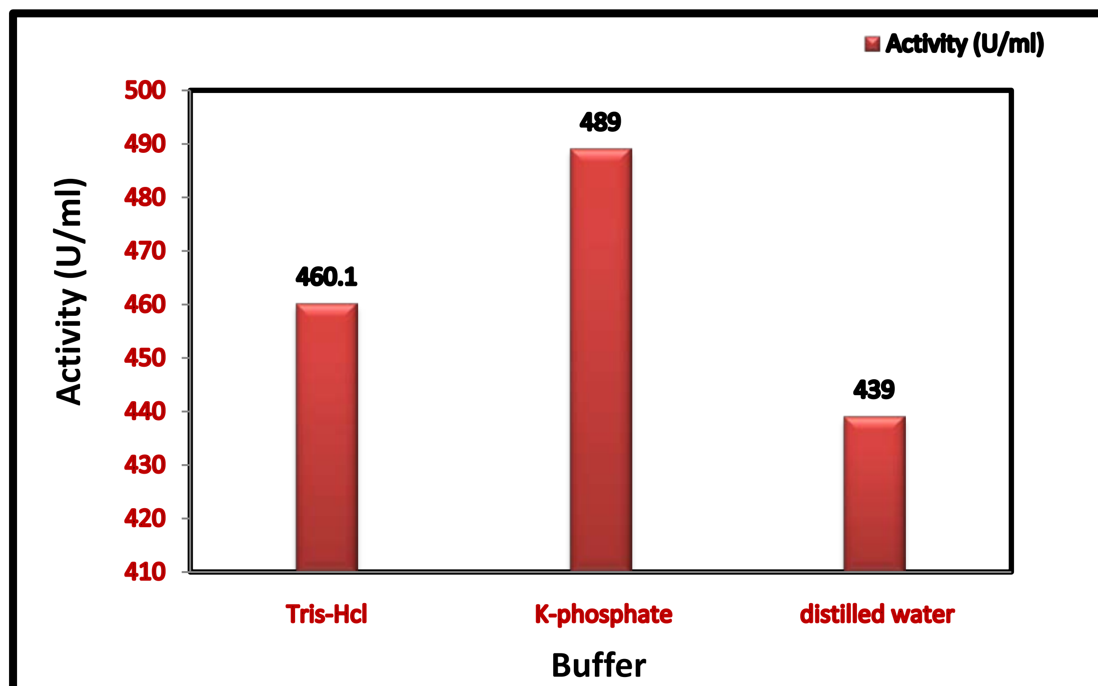


Figure (3-6): Effect of buffer solution pH8 on the activity of asparaginase extracted from seeds of *Pisum sativum* incubation at 37C for 30 min.

It has been reported that potassium phosphate buffer maintain a specific pH value (Mckee and Mckee, 1996).

3.3.6 Effect of enzyme:substrate ratio

Effect of enzyme:substrate ratio on the activity of asparaginase extracted from *Pisum sativum* seeds was studied by using different ratios of enzyme:substrate (1:1, 1:2, and 1:3) to determine the optimum for maximum asparaginase activity after incubation of enzyme with its substrate at these ratios under optimum conditions examined previously.

Results mentioned in figure (3-7) showed that asparaginase activity was reached 602.6 U/ml when the ratio of enzyme:substrate was 1:3. At this ratio, enzyme activity reaches

the steady state and doesn't affected with the increase of enzyme:substrate ratio.

It has been reported that enzyme assay was typically designed with the conception that measured activities will be directly proportional to the amount of active enzyme in the reaction mixtures until the optimum saturation ratio obtained with substrate.

Therefore the ratio 1:3 of enzyme:substrate was selected as the optimum for asparaginase activity. Arnnok *et al*., (2010) found that the increase in enzyme concentration results in linear increase in both enzyme activities for both of polyphenol oxidase and peroxidase extracted from *Capsicum annium* in an enzyme concentration ranged between 20 to 200µl and 25 to 100µl respectively.

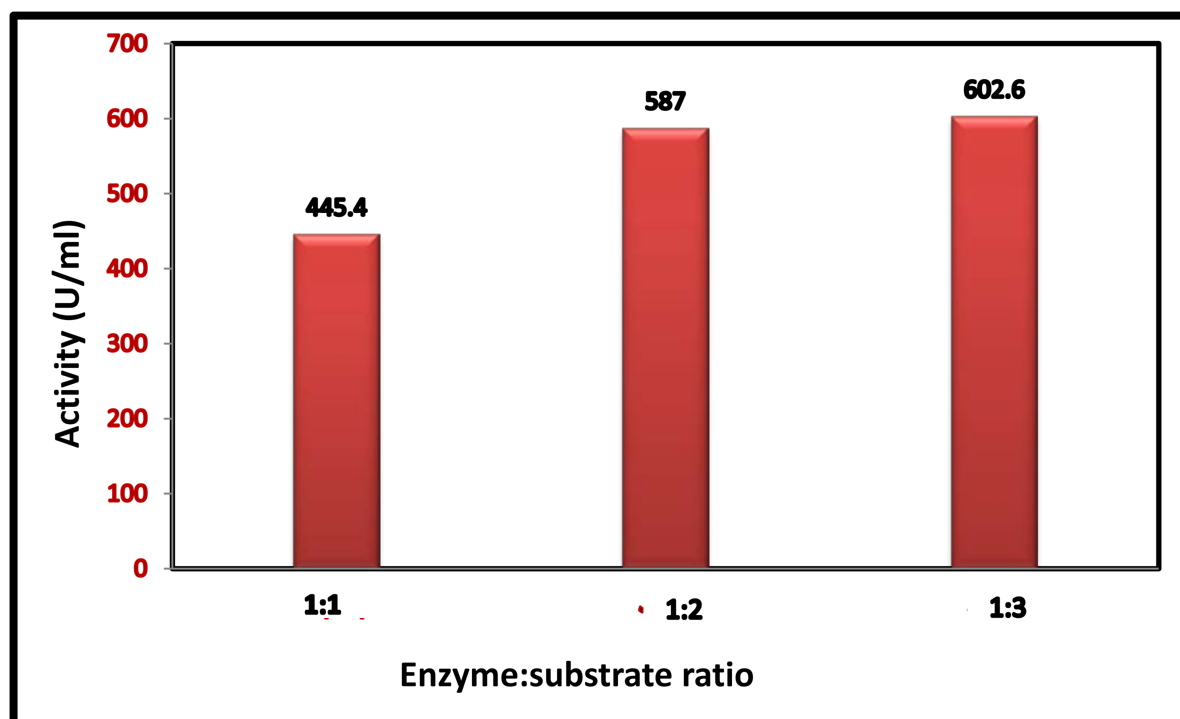


Figure (3-7): Effect of enzyme:substrate ratio on the activity of asparaginase extracted from seeds of *Pisum sativum*.

3.4 Purification of asparaginase extracted from seeds of *Pisum sativum*

Asparaginase in seed extracts of *Pisum sativum* was purified using two types of chromatography techniques includes ion-exchange and gel filtration chromatography. These techniques were used directly to purify asparaginase from crude extracts without the need to achieve the fractionation step with ammonium sulfate and dialysis step because it was found that the precipitation of asparaginase from seeds crude extracts using gradual saturation ratios of ammonium sulfate (35%, 40%, 45%, 50%, 55%, 60%, 65%, 70% respectively) cause a decrease in enzyme activity and specific activity in comparison with enzyme activity in crude extract as mentioned in table (3-3). These may be because of the susceptibility of asparaginase in seeds crude extracts to ammonium sulfate that interfere with enzyme activity even after dialysis step. According to these results, ammonium sulfate precipitation was excluded from the purification steps of *P. sativum* asparaginase.

On the other hand, dialysis of crude extract of asparaginase using dialysis bag against 0.05 M potassium phosphate buffer (pH 8.0) after 24 hours at 4°C doesn't have any significant increase in the activity or specific activity of the enzyme, the activity was 614.0 U/ml and the specific activity was 77 U/mg. Therefore dialysis step was neglected.

These findings were agreed with Majeed, (2011) who reported that ammonium sulfate precipitation of asparaginase from *W. sominfera* results in considerable loss of enzyme activity.

Table (3-3): Activity of asparaginase extracted from *Pisum sativum* seeds after precipitation with ammonium sulfate using different saturation ratios.

Saturation with ammonium sulfate (%)	Volume (ml)	Activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification fold	Yield (%)
35	20	312.2	7.7	40.5	6244	0.53	10.32
40	20	318.9	8.3	38.3	6378	0.50	10.54
45	20	312.3	7.9	39.5	6246	0.52	10.32
50	20	340.2	7.3	46.6	6804	0.61	11.24
55	20	215.4	5.3	40.6	4308	0.53	7.12
60	20	259	5.7	45.4	5180	0.83	8.56
65	20	300.3	5.2	60.1	6006	0.79	9.92
70	20	297.3	8.3	35.8	5946	0.47	9.82
Crude extract	100	605.0	8.0	75.6	60500	1	100

In other study, Mokrane, (2003) referred to same results when he mentioned that asparaginase produced by *E. coli* loss most of its activity at fractionation with ammonium sulfate (20-70%) saturation ratio.

3.4.1 Ion exchange chromatography

Asparaginase extracted from seeds of *Pisum sativum* was purified by ion-Exchange chromatography using DEAE-Cellulose an anionic exchanger. This matrix was used for purification because

it has high capacity for bioseparation, easy to prepare, multiple use, in addition to simplicity to separate different biomolecules (Karlsson *et al*., 1998). The choice of ion exchanger depends on the net charge of protein (asparaginase). According to results obtained from this study, optimum pH for asparaginase activity which was pH8, and the pI of this enzyme (pI=4.5-5) as described by (Chagaz and Sodek, 2001), it could be concluded that asparaginase extracted from seeds of *P. sativum* have negative charge.

Net charge= pI – pH Segal, (1976)

According to these findings, anionic exchanger DEAE-Cellulose was used for purification of asparaginase from *P. sativum.* A portion of 3 ml of crude asparaginase concentrated with sucrose was applied onto the surface of column gel matrix (DEAE-Cellulose). then column was washed and equilibrated with equal volume of 0.05 M potassium phosphate buffer solution (pH 8.0) to wash unbound proteins (uncharged and positively charged proteins) in asparaginase crude extract. The bound proteins (negatively charged) were then eluted using linear gradient concentrations of sodium chloride ranged between 0.1-0.5 Molar.

Results indicated in figure (3-8) showed that one protein peak appeared in the washing step, while there are four protein peaks were appeared after elution with gradient concentrations of sodium chloride. All these protein peaks in washing and elution steps were detected by measuring the absorbance at 280 nm for each eluted fraction. The four eluted proteins were assayed to detect asparaginase activity.

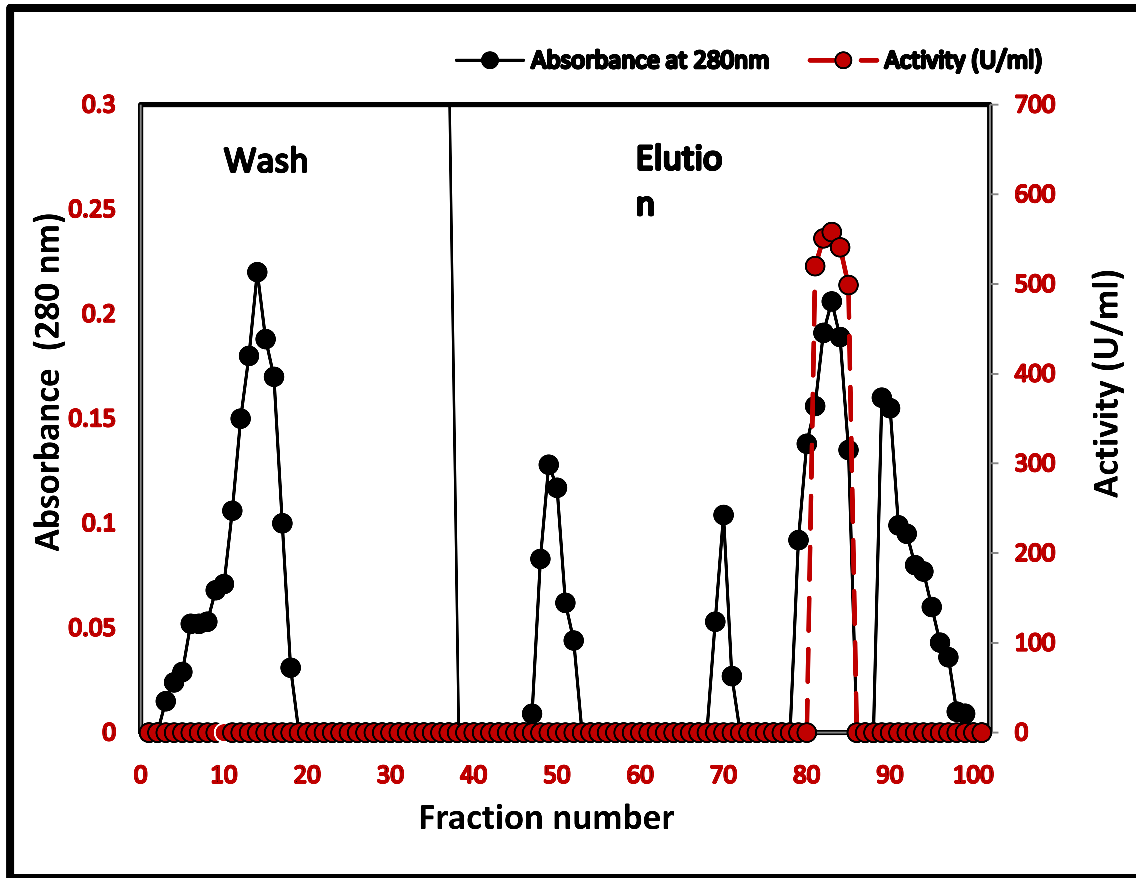


Figure (3-8): Ion exchange chromatography for purification of asparaginase produced from *Pisum sativum* seeds using DEAE-Cellulose column (2×23 cm) with a flow rate of 20 ml/hour.

Results illustrated in table (3-4) showed that the third peak that eluted in fraction numbers 80 to 84 has asparaginase activity that reaches 558.0 U/ml. Fractions represents asparaginase active were collected and pooled, then protein concentration, asparaginase activity, specific activity was estimated.

Results indicated in table (3-4) showed that the maximum asparaginase activity and specific activity in the asparaginase concentrate were 554.8 U/ml and 154.1 U/mg respectively, with 13.7% fold of purification.

In other study, ion exchange chromatography using DEAE-Cellulose was applied for purification of asparaginase from

Withenia sominfera (Majeed, 2011).

Partially purified asparaginase obtained from the ion exchange chromatography step was further purified by gel filtration chromatography technique. According to the dependence of ion exchange chromatography basically on charge difference principle, the presence of enzyme in the elution step confirmed that asparaginase produced by *Pisum sativum* was negatively charged.

3.4.2 Purification using gel filtration chromatography

Gel filtration chromatography technique was the next step used in the purification of asparaginase produced by *P. sativum* after purification by ion exchange chromatography technique. A volume of 5 ml of partially purified asparaginase was applied on Sephadex G-200 column (1.6×43 cm) which previously equilibrated with 0.1 M potassium phosphate buffer (pH 8.0). Sephadex G-200 has a separation limits ranging between (5000-600000 Dalton) which allows ability of separation with high degree of purification (Sivasankar, 2005). Furthermore, gel filtration is the simplest and mildest of all the chromatography techniques and separates molecules on the basis of differences in molecular size (Eli and Co, 2001b). Proteins were eluted thought the column matrix in a flow rate of 20 ml/hour. Protein peaks were detected by measuring the optical density at 280 nm using UV-VIS spectrophotometer. Results indicated in figure (3-9) showed that only one peak represents asparaginase activity was appeared after elution with potassium phosphate buffer. Fractions representing asparaginase activity were pooled, then protein concentration, asparaginase activity and specific activity were measured in 20 ml of enzyme concentrate

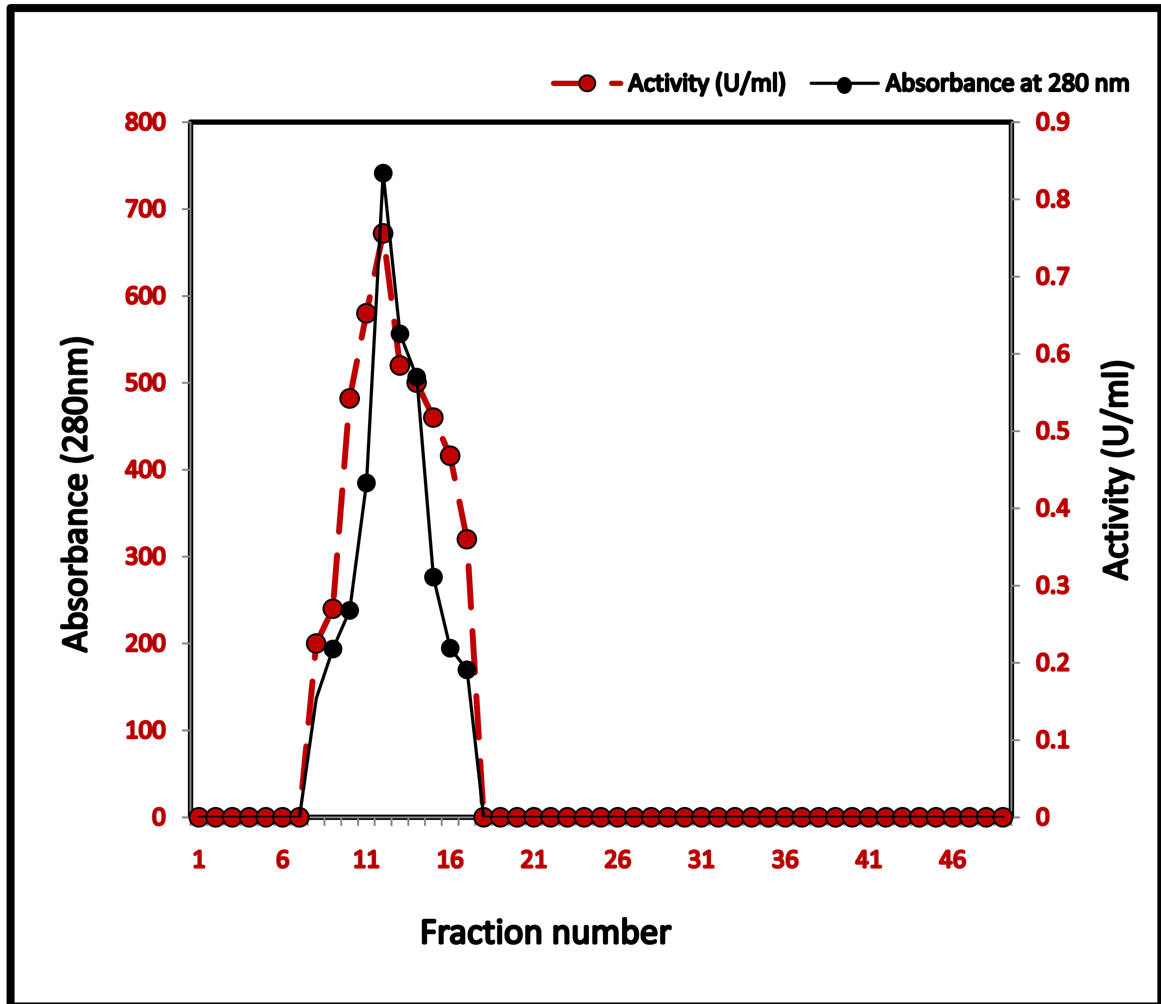


Figure (2): Gel filtration chromatography for purification of asparaginase extracted from *Pisum sativum* seeds using Sephadex G-200 column (1.6×43 cm) equilibrated with potassium phosphate buffer pH8.0, fraction volume was 5ml at flow rate of 20ml/hour.

Results indicated in table (3-4) showed that there is an increase in both activity of purified enzyme (732.4 U/ml) and specific activity (228.8 U/mg) with a purification fold 3.02 with an increase in the yield of asparaginase (24.2%).

In other study, El-Sayed *et al.*, (2011a) found that partial purification of asparaginase from chicken liver using Sephadex G-100 and Sephadex G-200 as a second step (after ammonium

sulfate precipitation) gives the highest specific activity of 158.11 U/mg protein and 128 fold of purification with 17% yield.

Dhevagi and Poorani, (2006) demonstrated that purification of asparaginase produced by *Streptomyces spPDK 2* using dialysis, precipitation with ammonium sulfate 45-85%, Sephadex G-50 gel filtration and Sephadex G-200 gel filtration gave a maximum specific activity of 63.0 U/mg with 82.9 fold of purification with only 2.18 % yield.

On the other hand, asparaginase from *Enterobacter aerogenes* purified by a simple method involving sonication of the crude cell mass, then by gel filtration with Sephacryl S-100 as the separating material, followed by ultrafiltration.

This procedure gives 10-fold of purification with a specific activity of 55 IU/mg protein and recovery of 54% (Mukherjee *et al*., 1999).

Table (3-4): Purification steps of asparaginase extracted from seeds of *Pisum sativum*.

Step	**Volume (ml)**	**Activity (U\ml)**	**Protein conc. (mg\ml)**	**Specific activity (U\mg)**	**Total activity (U)**	**Purification fold**	**Yield (%)**
Crude enzyme	100	605.0	8.0	75.6	60500	1	**100**
Ion exchange	15	554.8	3.6	154.1	8322	2.03	**13.7**
Gel filtration	20	732.4	3.2	228.8	14648	3.02	**24.2**

3.5 Characterization of purified asparaginase

3.5.1 Molecular weight of asparaginase

Molecular weight of the purified asparaginase produced by *P. sativum* was determined by gel filtration chromatography using Sephadex G-200 in the presence of four standard proteins which they are Pepsin (14000 Dalton), Trypsin (20000 Dalton), Bovine serum albumin (67000 Dalton), and Urease (430000 Dalton).

Asparaginase and each standard protein were applied and eluted individually. Results indicated in figure (3-10) showed that asparaginase has a molecular weight of 66,464 KeloDalton. Molecular weight of asparaginase differ according to the type of the produced genera, species and parts of an organism.

Sodek *et al*., (1980) reported that the molecular of the asparaginase from *P. sativum* seeds was 68 KDa using Sephadex LH-20 gel filtration technique. While Chagaz and Sodek, (2001) found that the molecular weight of asparaginase in the seeds of the same plant was 69 KDa using Sephacryl S-200 chromatography and also by mobility on native PAGE relative to BSA. There was no evidence for dissociation into subunits on SDS-PAGE, and this suggest that asparaginase is a monomeric protein of molecular weight 69 KDa.

On the other hand, Ireland and Joy, (1983) mentioned that the molecular weight of asparaginase produced from leaves of *P. sativum* was 58 KDa using hydroxylapatite chromatography.

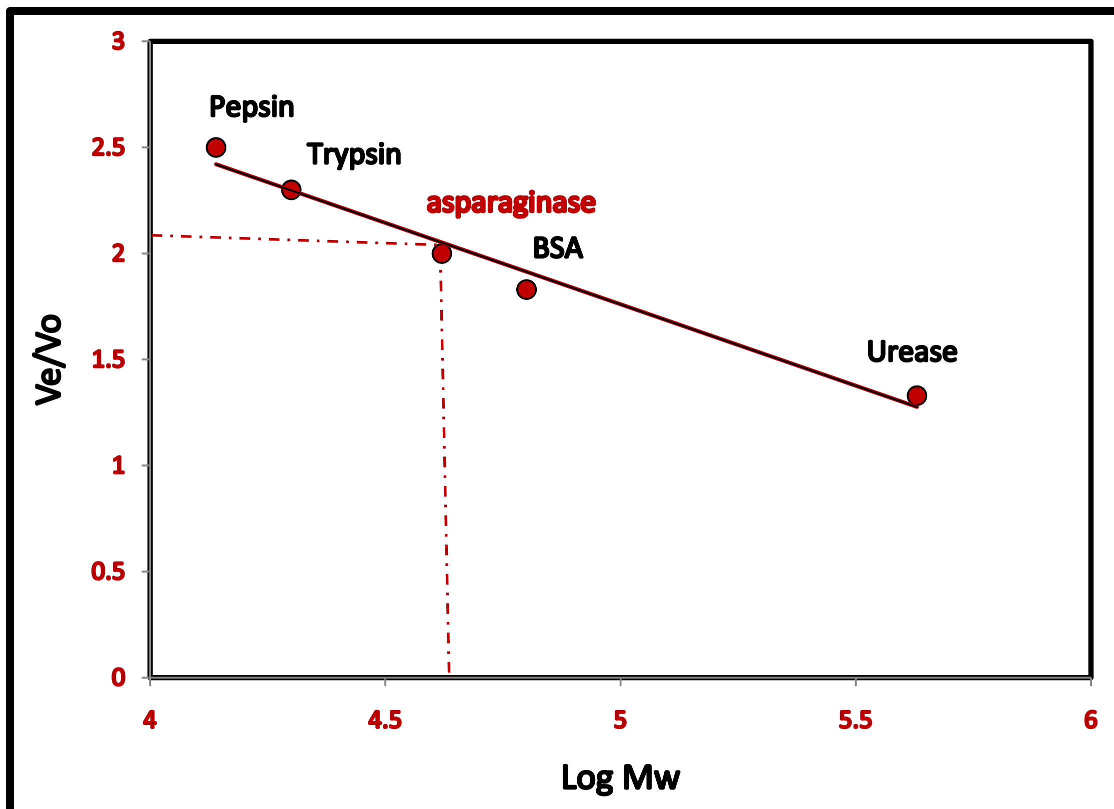


Figure (3-10): Selectivity curve for determining the molecular weight of purified asparaginase extracted from *Pisum sativum* seeds by gel filtration chromatography using Sephadex G-200 (1.6×43 cm).

3.5.2 Optimum pH for asparaginase activity

Optimum pH for asparaginase activity was determined by incubation of purified asparaginase with its substrate (asparagine) at different pH values ranging between pH4 and pH10.5.

Results indicated in figure (3-11) showed that asparaginase was active over a wide range of pH between 5 and 10 with an optimum activity of 632 U/ml and 622 U/ml at pH 8.5 and 8.0 respectively. These findings were in agreement with those of other studies which have shown that the optimum activity of asparaginase was obtained at pH 8.5 (Dunlop *et al*., 1978; Bano and Sivaramakrisnan, 1980 and Majeed, 2011).

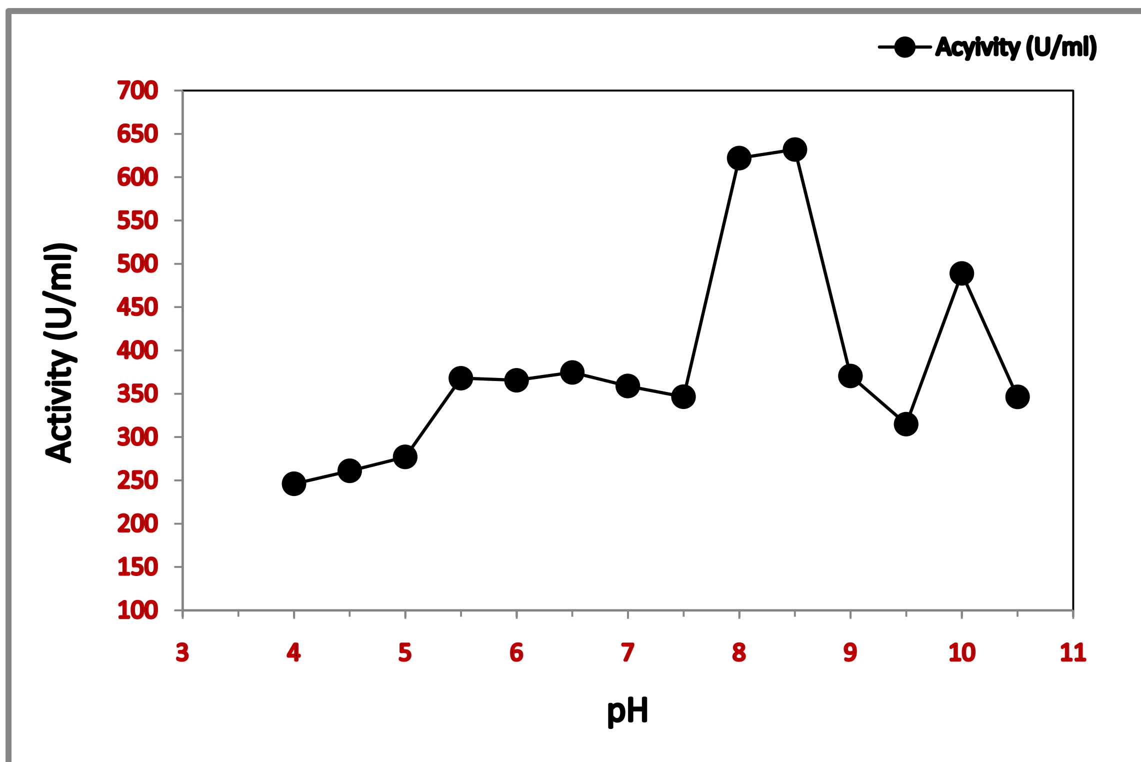


Figure (3-11): Effect of pH on activity of purified asparaginase extracted from seeds of *Pisum sativum*.

Effect of pH on the rate of hydrolysis of asparagine is due to the effect of asparaginase on the velocity of enzyme-substrate complex formation and breakdown, in addition to its effect on the ionic state of the active site of enzyme. The change in the hydrogen ion concentration may affect the ionization of side chains located in the active side (Lawrence, 1967; Bezkorvainy and Rafelson, 2007 and Mckee and Mckee, 1996).

Optimization of pH for asparaginase activity may vary depending on the different genera or species of an organism. *E.coli* has an optimum activity at pH8.6 (Zhang and Lynd, 2004), while the optimum pH for asparaginase activity of *Bacillus* spp. was in a pH 7.0 (Moorthy *et al*., 2010). This property of the enzyme make clear that the enzyme produced by *P. sativum* under the present study has effective carcinostatic property, because the

physiological pH is one of the perquisites for antitumor activity (Manna *et al*., 1995). Scheetz *et al*., (1971) reported that asparaginase activity below pH 8 would not be expected to be very effective for treatment of tumor patients.

3.5.3 Optimum pH for asparaginase stability

To determine the optimum pH for asparaginase stability, purified asparaginase was incubated at differtent pH values. The remaining activity was then determined after assaying enzyme activity.

Results indicated in figure (3-12) showed that asparaginase was more stable at pH 8.5 and 8.0 because at these pH values, enzyme gain maximum remaining activity (96% and 95% respectively), while the remaining activity was decreased when the enzyme was incubated at pH values less or more than optimum pH.

Whitaker and Bernard, (1972) found that most enzymes undergo irreversible denaturation in strong acidic or alkaline conditions.

Enzymes like other proteins, are stable over only a limited range of pH. Outside this range, changes in the charges on ionisable residues result in modifications of the tertiary structure of the protein and eventually cause denaturation (Fullbrook, 1983; Zubay, 1993).

Determination of pH on enzyme stability was important for the maintaining the enzyme conformation. This may differ among the kind of enzyme and the organism.

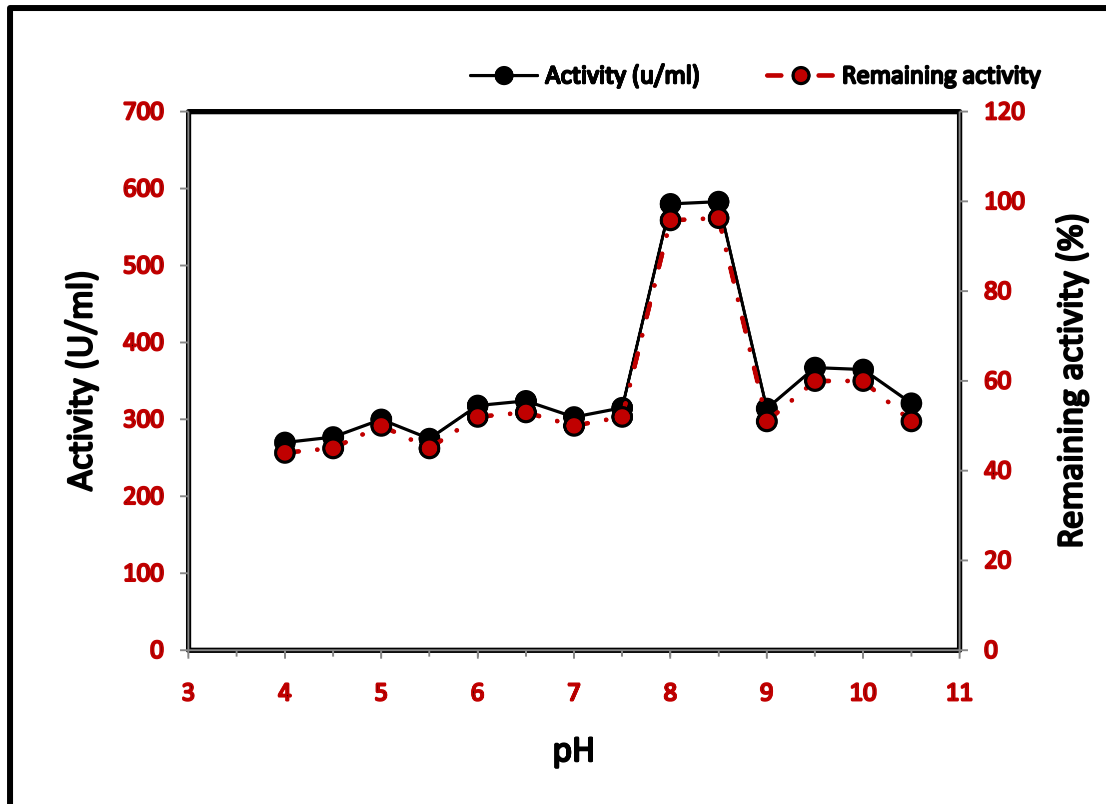


Figure (3-12): Effect of pH on stability of purified asparaginase extracted from seeds of *Pisum sativum*.

Stecher *et al.*, (1999) found that the tertrameric form of asparaginase stayed stable at pH ranged (4.5-11.5) with slight increase at alkaline pH which indicate a more stable conformation of the molecule. The asparaginase of *S. marcescens* was, however, most stable at the pH range of 5-9 (Aghaiypour *et al.*, 2001).

Enzyme that work either at extreme pH values or high temperature are now of biotechnological interest, therefore, purification of enzymes with these properties presents genuine commercial opportunities and a valuable contribution to the field of biotechnology.

3.5.4 Optimum temperature for asparaginase activity

Temperature is an important factor affects enzyme activity, the favorable temperature for asparaginase activity may differ with asparaginase sources. Therefore optimum temperature for asparaginase activity was determined, then enzyme activity and remaining activity was assayed at different temperatures.

Results indicated in figure (3-13) showed that 37°C was the optimum temperature for asparaginase activity, while enzyme activity was decreased at less or higher of this temperature. Most enzyme reactions were found to be accelerated with the increase in temperature for a limited range. Other studies indicated that optimum activity of asparaginase was obtained at 37°C (Roberts *et al*., 1968; Mohapatra *et al*., 1995; Sudarslal, 2000; El-Bessoumy *et al*., 2004 and Mohana *et al*., 2011).

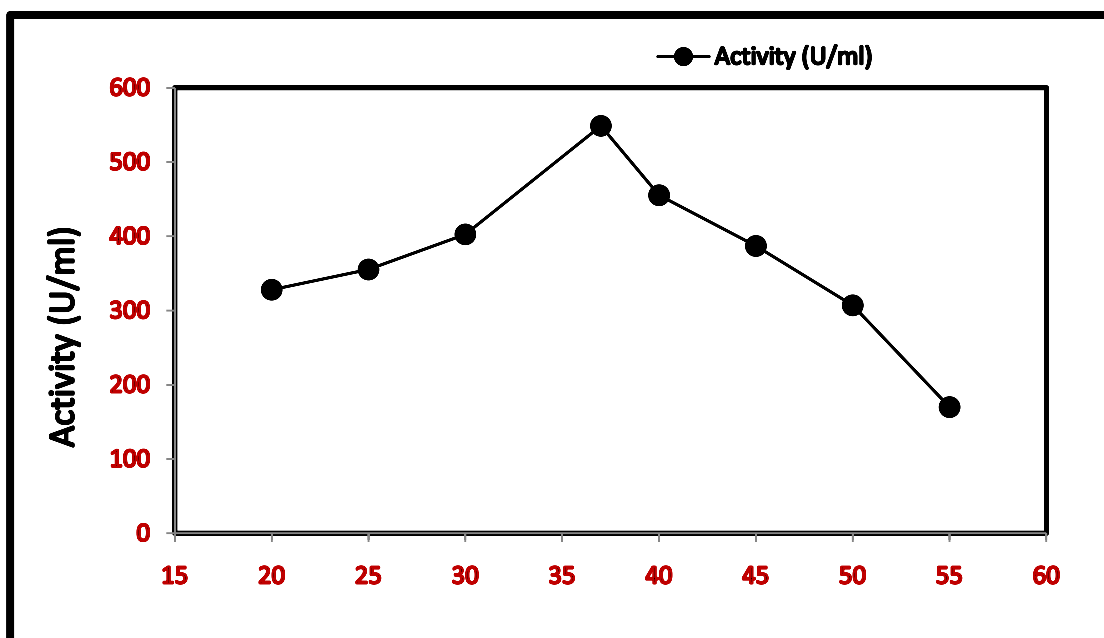


Figure (3-13): Effect of temperature on the activity of purified asparaginase extracted from seeds of *Pisum sativum*.

Despite that not all types of asparaginase were active at high temperature; asparaginase of *Aspergillus terresk* LS2 stayed active at 50°C (Siddalingeshwara and Lingappa, 2011) and at 80°C form *Bacillus* spp R36 (Hegazy and Moharm, 2010).

3.5.5 Optimum temperature for asparaginase stability

To determine the optimum temperature for asparaginase stability, enzyme was incubated at different temperature values, then enzyme activity and remaining activity was determined. Results indicated in figure (3-14) showed that asparaginase was more stable at 40°C because at this temperature enzyme gain maximum remaining activity (75%), then remaining activity decreased when the enzyme was incubated at temperature values more than the optimum for stability (40°C), and then asparaginase began to lose its activity after incubation at temperature 45°C. The decrease in the enzyme activity with the increase in temperature may lead to the denaturation of enzyme by destructing the three dimensional structure of protein and that cause a change in the active site which leads to inactivation of the enzyme at high temperatures. There is also a heat stable enzyme (from thermophiles organism) which may not be affected by high temperature due to its hydrophobic interactions and disulfide bonds which strengthen their structure (Price and Steven, 1982; Prescott *et al.*, 2005).

Segal, (1976) stated that crude and partially purified enzymes are more stable than purified enzymes due to the existence of carbohydrates and other proteins protecting them.

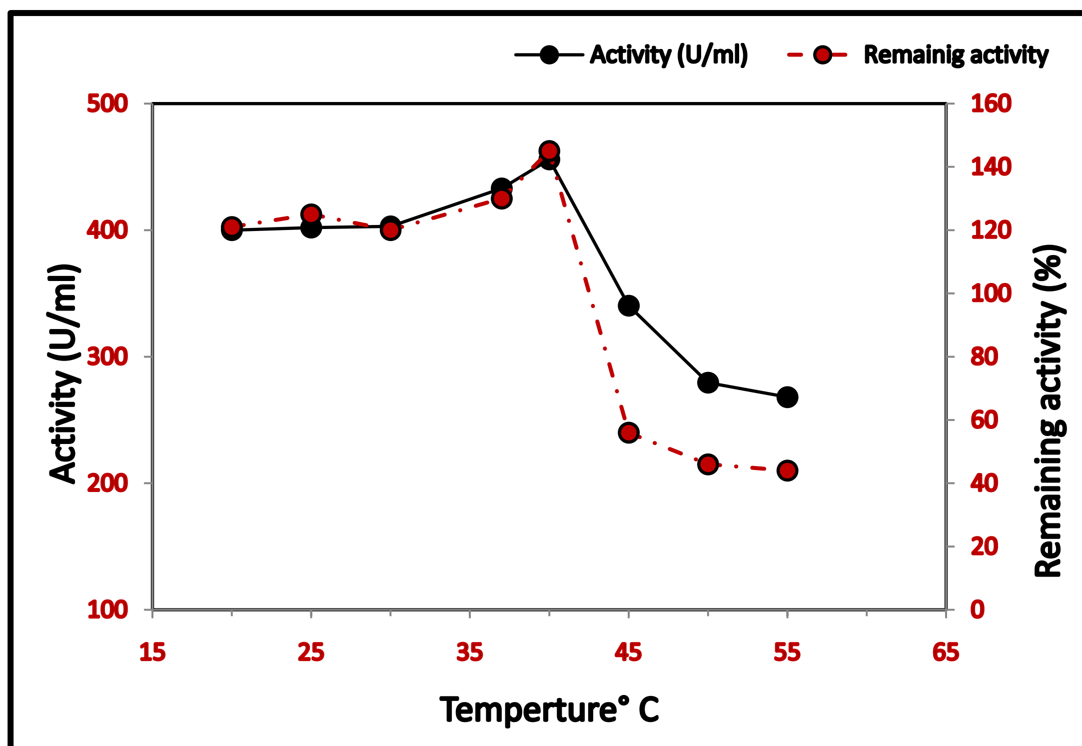


Figure (3-14): Effect of temperature on stability of asparaginase purified from seeds of *Pisum sativum*.

Dhevagi and Poorani, (2006) noticed that asparaginase of marine *Vibrio* lost approximately 20% of its activity through incubation at 60°C for 30 minutes.

On the other hand, Siddalingeshwara and Linappa, (2011) reported that asparaginase of *Aspergillus terres* KLS2 retained all of its activity through incubation at 70°C for 30 and 60 minutes.

3.5.6 Activation energy and Temperature Coefficient (Q_{10}) of asparaginase

Activation energy is the minimum energy required for the molecules to be converted to product (Whitaker and Bernard, 1972). Activation energy for asparaginase purified from seeds of

Pisum sativum was studied. Results indicated in figure (3-15) showed that the activation energy for asparaginase to convert asparagine to product by asparaginase was 6260 calories/mol.

In other study White *et al.*, (1973) reported that activation energy of chemical reactions ranged generally between 6000 and 15000 calories/mol, while Berg *et al.*, (2002) stated that whenever activation is low, the enzyme is more efficient in converting the substrate to product. These results are in consistent with Mokrane, (2003) who found that the activation energy of asparaginase II produced by *E.coli* was 6332 calories/mol. Activation energy of asparaginase of *Capsicum annum* was 3000 calories/mol. (Bano and Sivaramakrishnan, 1980) and that of asparaginase of *S. marcescens SA1* was 6194.1 calories/mol (Abdel Hameed, 2005).

Q_{10} is the Temperature Coefficient which represent the factor by which the reaction rate increases when the temperature is raised by ten degrees. The Q_{10} of asparaginase was measured to be 1.32. This result in agreement with Mokrane, (2003) who found that Q_{10} of *E.coli* asparaginase II was 1.43.

Q_{10} is unitless quantity. Q_{10} is a convenient way to examine and report the temperature dependence of the reaction rate, if the reaction rate is completely independent, it can be seen from the equation that the resulting Q_{10} will be 1.0.

If the reaction rate increases with increasing temperature, Q_{10} will be greater than 1.

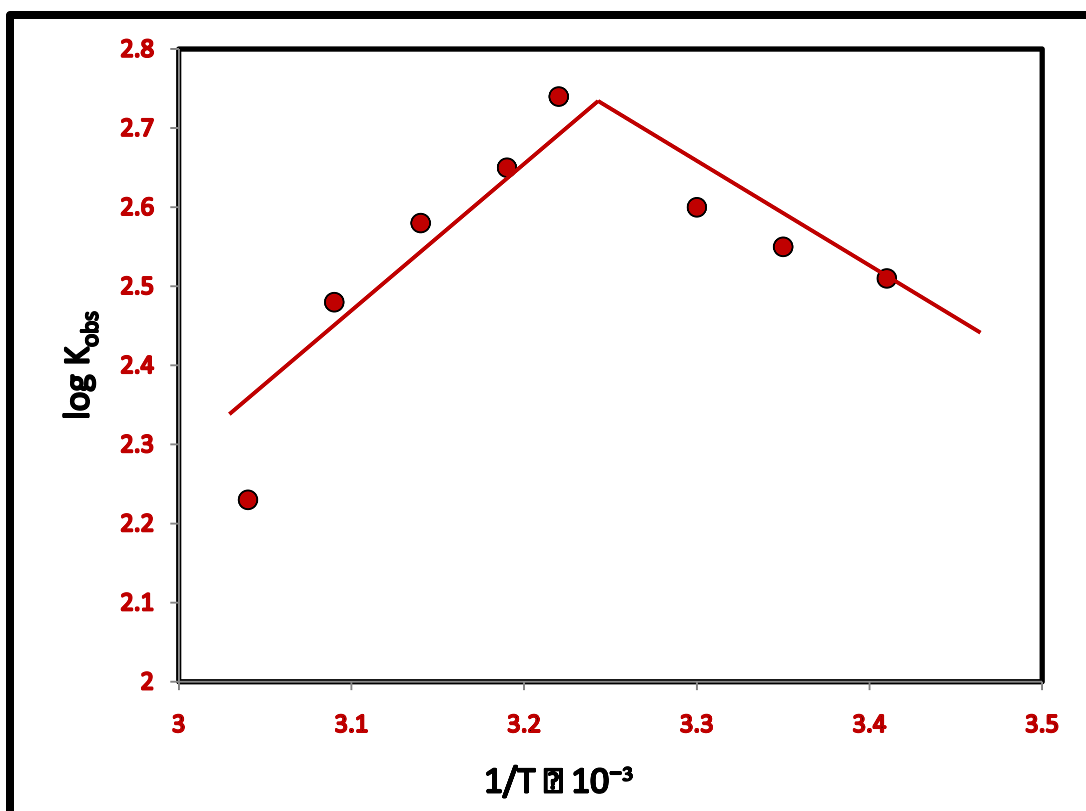


Figure (3-15): Arrhenius plot for determination the activation energy of asparaginase extracted from *Pisum sativum* seeds.

3.5.7 Enzyme specificity against different substrates

In order to investigate the specificity of asparaginase produced from *P. sativum* toward different substrates. Different substrates (L-asparagine, L-glutamine, L-aspartic acid and L-glutamic acid) were added to the purified enzyme at a concentration of 200 mM and the reaction mixture was incubated at 37°C for 30 minutes, then the activity and remaining activity were determined.

Results indicated in table (3-5) showed that the highest activity (747.2 U/ml) was obtained when L-asparagine was used as a substrate while less enzyme activity was recovered when L-glutamine, L-glutamic acid and L-aspartic acid were used. Asparaginase activity using these substrates was 47.6 U/ml, 36.1 U/ml, and 25.6 U/ml respectively. These results indicated that the

asparaginase extracted from *P. sativum* was more specific for asparagines as natural substrate.

This property of the enzyme is very essential in treatment of patients when incomplete removal of asparagines is required (Manna *et al*., 1995; Campbel *et al*., 1967).

In other study, Mokrane, (2003) found that asparaginase II from *E.coli* gives highest specificity against asparagines keeps 100% remaining activity in comparsion to L-glutamine, L-glutamic acid and D-L aspartic acid at a concentration of 0.01 mM that held remaining activity of 3.5%,0.0% and 0.0% respectively.

Table (3-5): Specificity of asparaginase purified from *P. sativum* against different substrates.

Substrate	Substrate Conc. (mM)	Activity (U/ml)
L-asparagine	200	**747.2**
L-aspartic acid	200	**25.6**
L-glutamine	200	**47.6**
L-glutamic acid	200	**36.1**

Basha *et al*., (2009) revealed that asparaginase from marine actinomycetes have also a substrate specificity towards L-asparagine. Asparaginase from *Capsicum annum* has inseparatable glutaminase activity and was asparaginase-gltaminase enzyme towards L-asparagine, L-glutamine, D-asparagine and D+L-asparagine (100%, 50%, 7% and 23% respectively).

While Davidson *et al*., (1977) revealed that asparaginase-glutaminase from *P. acidovorans* hydrolyze L-glutamine in 150 % of their ability to hydrolyze L-asparagine (100%).

3.6 Cytotoxicity of asparaginase on tumor cell line

The cytotoxicity effect of purified asparaginase extracted from *P. sativum* on tumor cell line was studied by evaluating its effect on L20B cell line (passage 18) on exposure time of 48 hours at various concentrations of asparaginase (150.0, 75.0, 37.5, 18.75, 9.37, 4.68, 2.34 and 1.17µg/ml) using Neutral Red assay. The optical density measured at transmitting wave length 450nm and 492nm.

The Neutral Red assay is a cell survival/viability assay based on the ability of viable cells to incorporate and bind Neutral Red dye. The Neutral Red uptake assay provides one of the most used cytotoxicity tests with many biomedical and environmental applications. Therefore it was selected for determination of asparaginase cytotoxicity (Winckler,1974; Guillermo *et al*., 2008).

Following exposure to enzyme, cells are incubated in the presence of Neutral Red dye. The dye readily penetrates cell membranes and accumulates intracellularly in lysosomes. As Neutral Red is a vital stain, it was used for staining living cells.

Changes of the cells brought about by the action of the purified asparaginase causes a decreased in the uptake and binding of Neutral Red. After washing cells with PBS and treating with destaining solution to release any excess of dye taken up, the level cells damage was evaluated by measuring the optical density of treated cell solution and comparing it to untreated negative control samples. Microtiter plate reader equipped with 450 and 492nm filter.

Results indicated in figure (3-16) showed that asparaginase showed gradual decrease in the viability of the cells in comparison with negative controls.

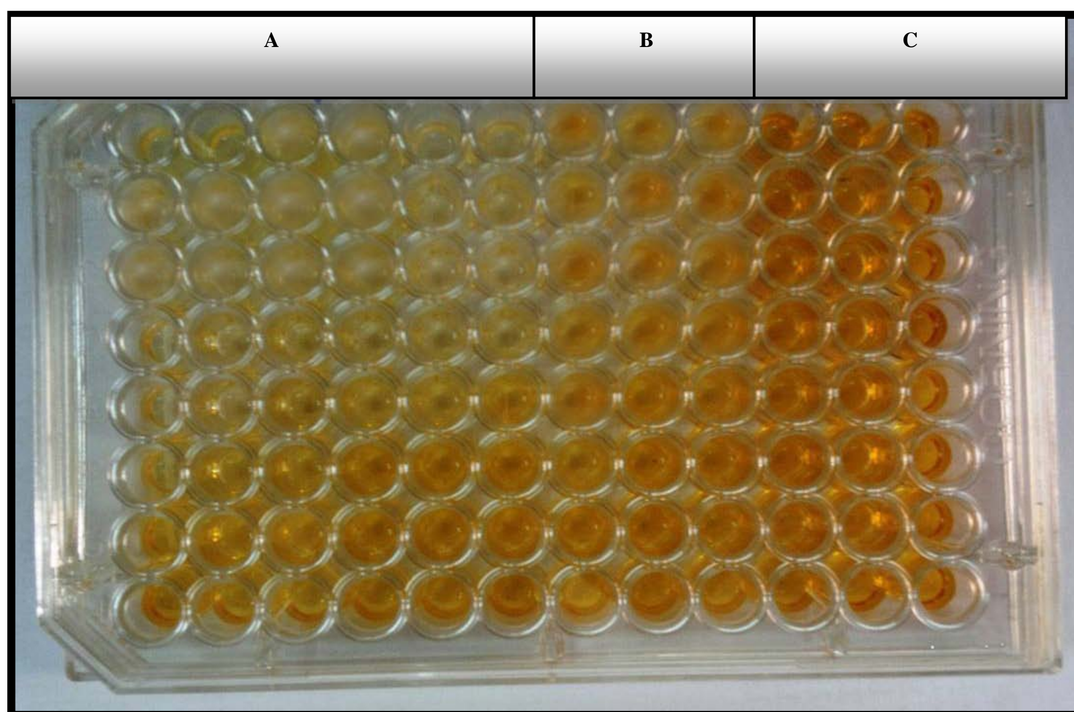


Figure (3-16): Cytotoxicity of purified asparaginase from *P. sativum* seeds against L20B cell line using Neutral Red assay.

A: Test sample: the L20B cell line after treatment with asparaginase (six times duplicate at each concentration)

B: Blank : the L20B cell line after treatment with only phosphate buffer saline

C: Negative control : the L20B cell line without any treatment

The 96-microtiter plate was used as a standared tool for cytotoxicity analysis, as indicated in Appendex (1), which viewed the example map (Plate configuration) of microtiter plate.

The cytotoxicity effect depends on the percentage of inhibition growth rate (I.R) which represents the cytotoxicity of asparaginase. Remaining activity percentage and cell survival percentage were estimated.

Results indicated in figure (3-17) showed that asparaginase has significant cytotoxicity effect on L20B cell line in concentration range between 150 µg/ml to 1.17 µg/ml in comparison with the negative control (the same cell line without any treatment) and blank (the same cell line treated only with phosphate buffered saline).

Results indicated in figure (3-17) showed that growth inhibition of tumor cell line was increased gradually with the increase of asparaginase concentration treated with. Maximum inhibitory effect of asparaginase was reached 39% growth when the cell culture of L20B was treated with 75µg/ml of asparaginase, then the inhibitory effect was decreased to 23% after treatment with 150 µg/ml of asparaginase.

On the other hand, results indicated in figure (3-18) showed that the inhibitory effect of asparaginase against L20B tumor cell line according to the growth inhibition results determined at 492 nm after treatment of this cell line with asparaginase concentrations was ranged between 150-1.17 µg/ml for 48 hours.

The inhibitory effect was increased with the increase in enzyme concentration and reaches the maximum (34.3%) after the

treatment with purified asparaginase at a concentration of 75 µg/ml, then the inhibitory effect was decreased to 24% after the treatment with 150 µg/ml of asparaginase.

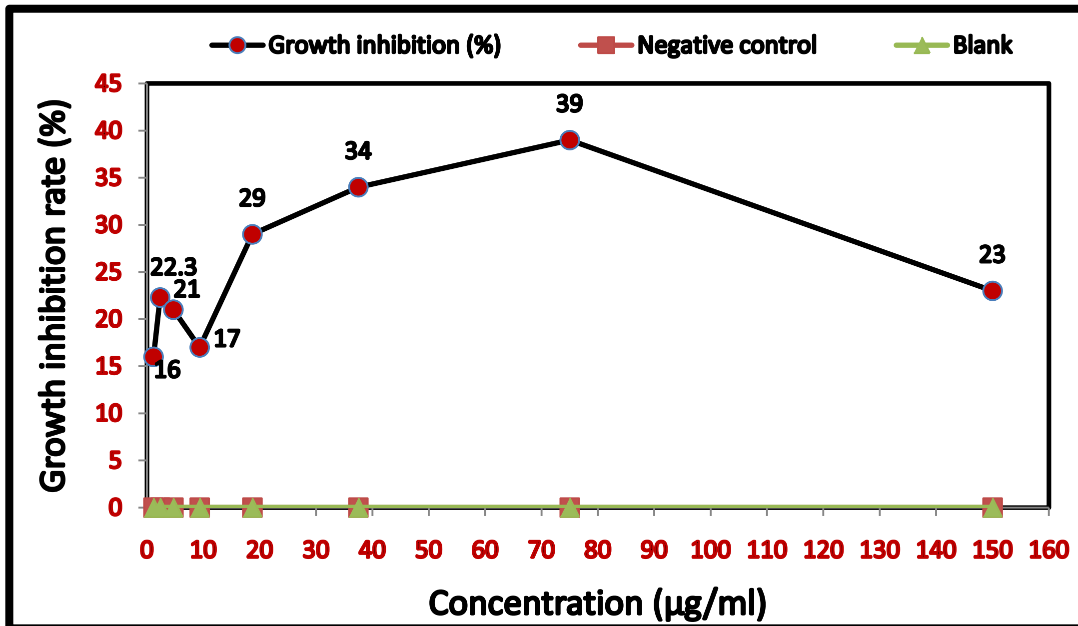


Figure (3-17): Cytotoxicity effect of purified asparaginase extracted from seeds of *Pisum sativum* on L20B cell line after incubation period for 48 hours at 450nm.

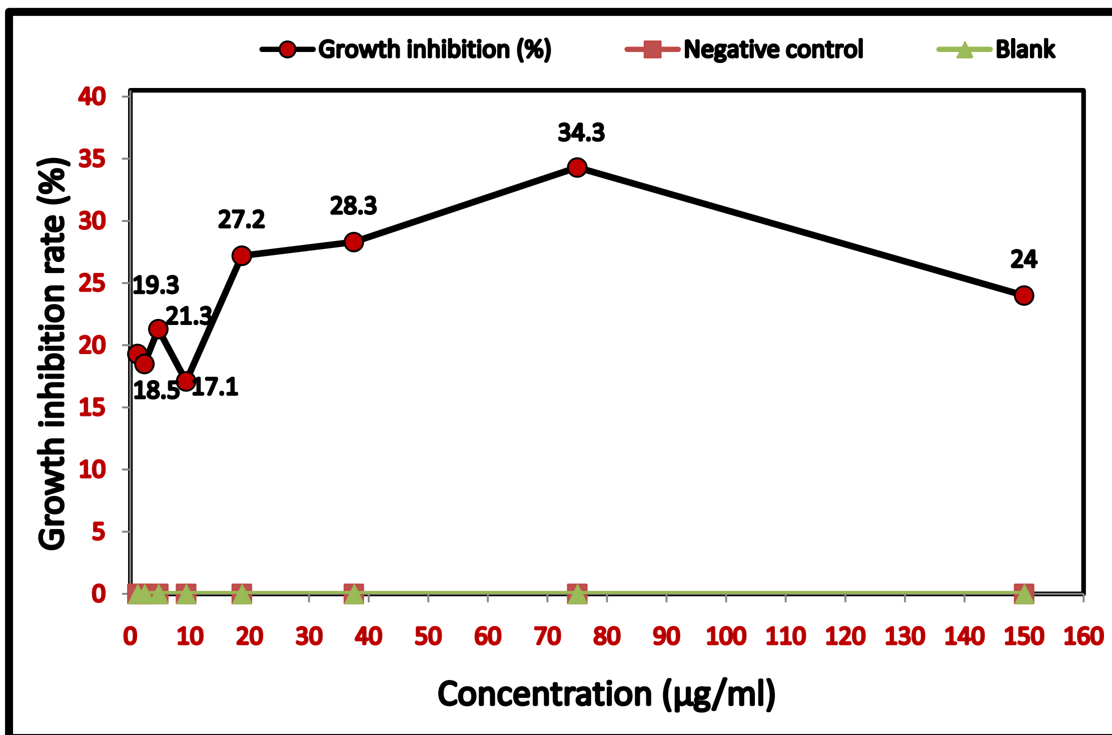


Figure (3-18): Cytotoxicity effect of purified asparaginase extracted from seeds of *Pisum sativum* on L20B cell line after incubation period for 48 hours at 492 nm.

Cell survivals (%) and remaining activity (%) of L20B tumor cell line after treatment with purified asparaginase (for 48 hours) was depends on the concentration of asparaginase at both wave lengths (450 and 492nm).

Results indicated in table (3-6) also showed that cell survivals of L20B cell line at 450nm was highly reduced after incubation with asparaginase at concentrations 4.68 and 37.5 µg/ml, at these concentrations, the survivals of L20B cell line were 70.8% and 72.7% respectively, while the cell line remaining activity percentage was reduced to 60.7% and 65.9% after incubation with asparaginase concentration of 75 and 37.5 µg/ml respectively.

Table (3-6): Cytotoxicity effect of different concentrations of purified asparaginase from *Pisum sativum* seeds on L20B tumor cell line after incubation for 48 hours measured at 450nm.

Asparaginase concentration (µg/ml)	Remaining Activity of cells (%)	Cell survival (%)
150	76.9	74.8
75	60.7	73.2
37.5	65.9	72.7
18.75	70.2	73.0
9.37	82.2	74.5
4.68	78.9	70.8
2.34	77.6	75.9
1.17	83.3	75.2

On the other hand, results indicated in table (3-7) showed that cell survivals (%) of L20B tumor cell line measured at 492 nm after 48 hour of treatments with asparaginase reaches the maximum reduction (72.0%) after treatment with asparaginase at a concentration of 4.68 µg/ml, while the remaining activity of tumor cells was 71.6% at concentration 37.5 µg/ml.

Table (3-7): Cytotoxicity effect of different concentrations of purified asparaginase from *Pisum sativum* seeds on L20B tumor cell line after incubation for 48 hours measured at 492nm.

Asparaginase concentration (µg/ml)	Remaining activity of cells (%)	Cell survival (%)
150	75.7	**74.5**
75	65.6	**76.1**
37	71.6	**75.5**
18	73.0	**75.3**
9.35	85.4	**77.5**
4.68	78.6	**72.0**
2.34	81.4	**81.2**
1.17	80.6	**77.1**

Activity of asparaginase was determined in cell suspension of L20B cell line after the treatment with asparaginase at concentration 75 µg/ml for 48 hours of incubation. According to these results, the concentration 75 µg/ml of asparaginase was selected due to its highly inhibitory effect on L20B tumor cell line (39%) as mentioned in figure (3-19).

On the other hand, results mentioned in figure (3-19) indicated that asparaginase activity decreased from 650 U/ml to 320 U/ml after 48 hours of the treatment, this may be due to the depletion of enzyme substrate (asparagines) into aspartate and ammonia that preventing tumor cells proliferation and promotion.

Grigoryan *et al*., (2004) reported that any decrease in the activity of asparaginase accompanied by a decrease in the asparagine level in the blood of leukemia patients. Mauz-Korholz *et al*., (2000) also reported that the depletion of extracellular asparagine levels was believed to be the major mechanism of cytotoxicity *in vitro*, because cancer cell but not normal cell, have an asparagine synthetase deficiency. Thus cancer cells essentially depend on the supply of L-asparagine.

Other findings noticed by Teerayat *et al*., (2009) showed that asparaginase has no significant effect on normal cell line after 48 hours of incubation, while Oza *et al*., (2009) found that asparaginase from *W. sominifera* has slight antileukemia effect on leukemic cell line. In general, asparaginase can exert immunsuppressive effects. Chakabarti and Friendman, (1970) using animal model demonstrated the depression of both humoral and cellular immune reactions by asparaginase.

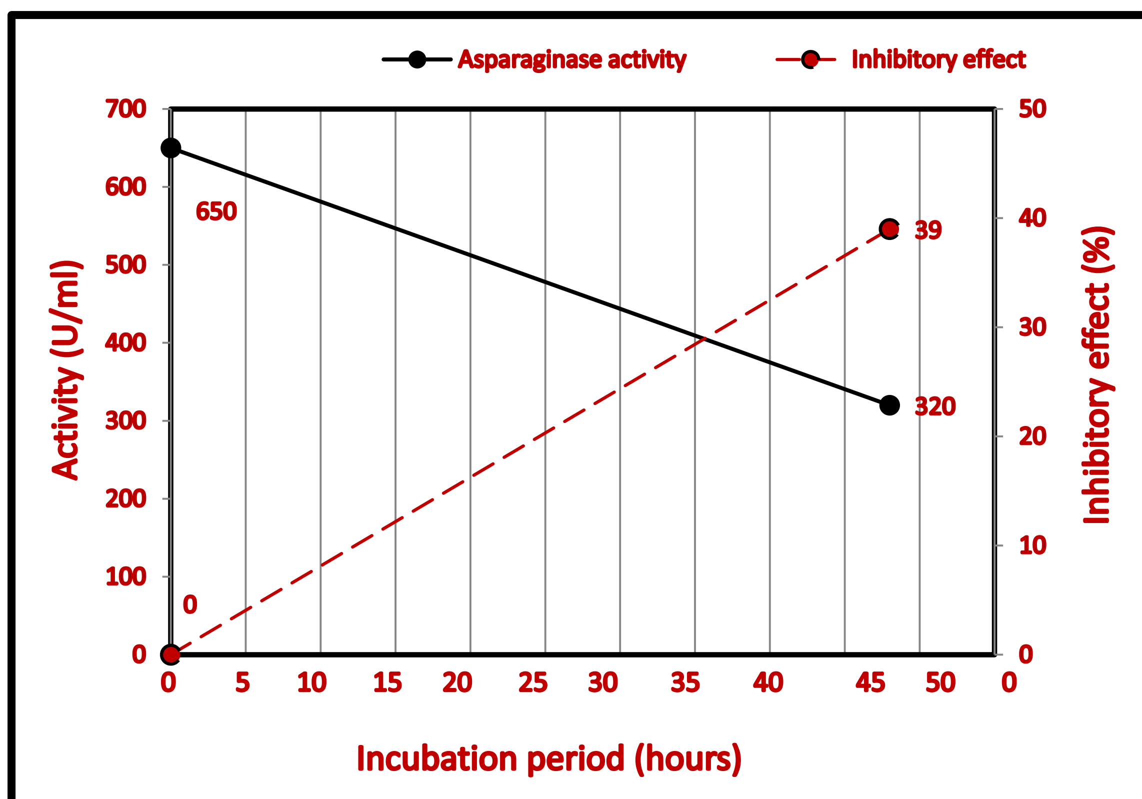


Figure (3-19): Activity of asparaginase purified from *Pisum sativum* and its inhibitory effect on L20B tumor cell line after incubation for 48 hours.

Conclusions and Recommendations

Conclusions:

1- Asparaginase isolated from different parts of *P. sativum* subspecies *Jof* proved to be efficient source for asparaginase.

2- *P. sativum* seeds was the best source for asparaginase production than other plant parts.

3- Optimum conditions for the activity of crude asparaginase includes the incubation of asparaginase with its asparagines substrate at a concentration of 200mM in a ratio (1:3) in presence of 0.05M potassium phosphate buffer (pH8) at 37°C for 30 minutes.

4- Asparaginase produced by *P. sativum* was sensitive to ammonium sulfate precipitation (35-70%).

5- Asparaginase extracted from seeds of *P. sativum* can be purified easily by using ion exchange and gel filtration chromatography techniques.

6- Purified asparaginase has a high molecular weight 66,464 Kelodalton, and was active and stable at pH 8.5 at 37°C and 40°C respectively, Activation energy was 6260 calories/mole.

7- Purified asparaginase has a cytotoxic effect against L20B tumor cell line.

Recommendations:

1- Studying the productivity of asparaginase from *P. sativum* at different stages of growth.

2- Increase the production of asparaginase from *P. sativum* by using plant tissue culture techniques.

3- Studying the Kinetic of asparaginase extracted from the *P. sativum*.

4- Immobilization of asparaginase using different methods for applicable purposes.

5- Determine the cytotoxicity effect of purified asparaginase towards different cell lines for different periods of incubation.

6- Studying the anti-leukemic activity of purified asparaginase *in vivo* and *in vitro*.

7- Using asparaginase in biosensors for monitoring the asparagine levels in mammalian and hybridoma cells.

8- Studying amino acid sequence of the novel asparaginase extracted from *P. sativum* and alignment with other asparaginase using protein database for molecular comparison.

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Appendix

1.1 Appendix: Example map of microtiter plate that describe the treatment

	1	2	3	4	5	6	7	8	9	10	11	12
A	TS (150 µg/ml)	TS (150 µg/ml)	TS (150 µg/ml)	TS (150 µg/ml)	TS (150 µg/ml)	TS (150 µg/ml)	B (5 µg/ml)	B (5 µg/ml)	B (5 µg/ml)	NC	NC	NC
B	TS (75µg/ml)	TS (75µg/ml)	TS (75µg/ml)	TS (75µg/ml)	TS (75µg/ml)	TS (75µg/ml)	B (2.5 µg/ml)	B (2.5 µg/ml)	B (2.5 µg/ml)	NC	NC	NC
C	TS (37.5 µg/ml)	TS (37.5 µg/ml)	TS (37.5 µg/ml)	TS (37.5 µg/ml)	TS (37.5 µg/ml)	TS (37.5 µg/ml)	B (1.25 µg/ml)	B (1.25 µg/ml)	B (1.25 µg/ml)	NC	NC	NC
D	TS (18.75 µg/ml)	TS (18.75 µg/ml)	TS (18.75 µg/ml)	TS (18.75 µg/ml)	TS (18.75 µg/ml)	TS (18.75 µg/ml)	B (0.62 µg/ml)	B (0.62 µg/ml)	B (0.62 µg/ml)	NC	NC	NC
E	TS (9.37 µg/ml)	TS (9.37 µg/ml)	TS (9.37 µg/ml)	TS (9.37 µg/ml)	TS (9.37 µg/ml)	TS (9.37 µg/ml)	B (0.31 µg/ml)	B (0.31 µg/ml)	B (0.31 µg/ml)	NC	NC	NC
F	TS (4.68µg/ml)	TS (4.68µg/ml)	TS (4.68µg/ml)	TS (4.68µg/ml)	TS (4.68µg/ml)	TS (4.68µg/ml)	B (0.15 µg/ml)	B (0.15 µg/ml)	B (0.15 µg/ml)	NC	NC	NC
G	TS (2.34µg/ml)	TS (2.34µg/ml)	TS (2.34µg/ml)	TS (2.34µg/ml)	TS (2.34µg/ml)	TS (2.34µg/ml)	B (0.07 µg/ml)	B (0.07 µg/ml)	B (0.07 µg/ml)	NC	NC	NC
H	TS (1.17 µg/ml)	TS (1.17 µg/ml)	TS (1.17 µg/ml)	TS (1.17 µg/ml)	TS (1.17 µg/ml)	TS (1.17 µg/ml)	B (0.03 µg/ml)	B (0.03 µg/ml)	B (0.03 µg/ml)	NC	NC	NC

TS: Test Sample (L20B cell line treated with purified asparaginase)

B: Blank (L20B cell line treated with PBS)

NC: Negative control (L20B cell line without any treatment)

الخلاصة

جمعت عينات من نبات البزاليا *Pisum sativum* من حقول زراعية في كلية الزراعة / قسم البستنة جامعة بغداد و شخصت على انها *Pisum sativum* subspp. *Jof* وفقا للصفات المظهرية للنبات . تم الكشف عن فعالية انزيم الاسباراجينيز في مستخلصات بذور و سيقان و اوراق النبات وقد أشارت النتائج أن أعلى فعالية للأسباراجينيز كانت في مستخلصات البذور النباتية.

تم التحري عن فعالية أنزيم الأسباراجينيز في مستخلصات البذور والسيقان و الأوراق النباتية وقد أشارت النتائج الى ان اعلى فعالية للأنزيم كانت في مستخلصات البذور اذ بلغت 30 وحدة /مل مقارنة ب 26.4 و16.1 وحدة /مل في مستخلصات الأوراق و السيقان النباتية على التوالي و على هذا الأساس فقد تم أختيار البذور النباتية مصدرا لأنتاج و توصيف الأنزيم ودراسة فعاليته ضد السرطانية.

درست الظروف المثلى لفعالية أنزيم الأسباراجينيز الخام المستخلص من البذور النباتية وقد أشارت النتائج الى أن أعلى فعالية للأنزيم قد تحققت بحظن الأنزيم الخام مع الأسباراجين بتركيز 200mM بنسبة 1:3 في محلول فوسفات البوتاسيوم المنظم بتركيز 0.05 مولار برقم هيدروجيني pH8 لمدة 30 دقيقة بدرجة 37م°. اذ بلغت الفعالية الانزيمية للاسباراجينيز 602.6 وحدة/ملغم تحت هذه الضروف.

تم تنقية الاسباراجينيز الخام المستخلص من البذور النباتية بخطوتين تضمنت الاولى التنقية بكروماتوغرافيا التبادل الايوني باستخدام مبادل الايونات الموجبة DEAE-Cellulose ثم التنقية بكروموتوغرافيا الترشيح الهلامي باستخدام السيفاديكس G-200 وقد بلغت الفعالية النوعية للانزيم المنقى 228.8 وحدة /ملغم.

تم توصيف انزيم الاسباراجينيز المنقى من بذور البزاليا , اذ بلغ الوزن الجزيئي للأنزيم 66,464 كيلو دالتون وكان الرقم الهيدروجيني الأمثل لفعالية وثبات الانزيم هو pH8.5 في حين كانت الدرجة الحرارية المثلى لفعالية وثبات الانزيم هي 37م° و 40م° على التوالي , وقد بلغت الطاقة الحرارية الازمة لتنشيط الانزيم 6260 سعرة/مول, أما قيمة المعامل الحراري (Q_{10}) للانزيم فقد بلغ 1.32.

درست الفعالية ضد السرطانية لانزيم الاسباراجينيز المنقى من بذور البزاليا باستخدام الخط الخلوي L20B وذلك بحضنه مع تراكيز متدرجة من الانزيم لمدة 48 ساعه وقد أشارت النتائج الى وجود فعالية تثبيطية لنمو الخلايا السرطانية تزداد بزيادة تركيز الانزيم مقارنة بخلايا السيطرة.

بسم الله الرحمن الرحيم

صدق الله العظيم

سورة النور

أية ٣٥

الى امي .... حنانا وعطفا ورحمة ....

وبعضا من الوفاء.......

الى سندي ومعلمي ...... ابي

الى اخي الغالي.... ونور عيني اختي نور....

اهدي ثمرة جهدي المتواضع

زينة

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الأحيائية

استخلاص وتنقية انزيم الاسباراجينيز المعزول من نبات البزاليا *Pisum sativum* ودراسة سميته ضد الخط السرطاني L20B

رسالة

مقدمة الى كلية العلوم - جامعة النهرين وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الأحيائية

مِن قبل

زينة عبداللة خلف

بكالوريوس تقانة احيائية / جامعة النهرين (2009)

ربيع الثاني 1433

اذار 2012