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The influence of some amino acids,vitamins and anti-inflammatory drugs on activity of chondroitinase produced by *Proteus vulgaris* caused urinary tract infection

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Abstract

Two hundred and ten specimens include urine, blood and ear swab were collected from different hospitals in Baghdad city; 85 (40%) isolates were diagnosed as *Proteus spp*. with (82%), (11.7%) and (5.8%) represented in urine, blood and ear swab specimens respectively. PCR technique was shown 30 (35.3%) isolates were positive for specific *Urease C* gene that used in rapid detection of *Proteus vulgaris*. The ability for chondritinase production was checked *invetro* and *invevo*, 24 (80%) isolates of *P. vulgaris* were showed ablity to chondritinase production and the isolate (p17) has higher enzyme activity value to (175.2U/ml). The Chondroitinase was purified by three short steps only included precipitate with 60% saturated of ammonium sulfate, dialysis and then filtrated by Sephadex G-150. The enzymatic activity was peaked to (270U/ml) and specific activity (692.3U/mg). The enzymatic activity for purified enzyme was increased significantly ($P<0.01$) to (563.7U/ml) after treatment with 1M of tyrosine but aspartic acid and glutamic acid were show inhibitory effect on enzyme activity decreased ($P<0.01$) to (130.U/ml) and (147.3 U/ml) respectively. The enzymatic activity was also increased significantly ($P<0.01$) reached to (633.6U/ml) and to(610.3U/ml) when treated with 50% of vitamin k and piroxicam respectively. This study was aimed to investigate effect of amino acids, vitamins and some nonsteroidal anti-inflammatory drugs (NSAIDs) with different concentrations on enzyme activity.

Keywords: Urease C, *Proteus vulgaris*, Chondroitinase, Tyrosine,Vitamin k, Feldene.

تأثير بعض الأحماض الأمينية والفيتامينات وبعض العقاقير المسكنة للألام على فعالية انزيم ال chondroitinaseالمنتج من بكتريا *proteus vulgaris* المسببه لألتهاب المسالك البولية

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الخلاصة

تم جمع مائتين وعشرة عينات من البول والدم ومسحات الاذن من مختلف المستشفيات في مدينة بغداد. تم تشخيص(85عزلة) من بكتريا *Proteus spp.* وبنسبة (40%) وشكلت عزلات ال*Proteus spp* نسب (82%) من عينات البول و(11.7%) دم و(5.8%) من مسحات الاذن على التوالي .اظهر التشخيص

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بتقنية تفاعل البلمرة التسلسلي PCR وجود (30 عزلة) تابعة لبكتريا ال*Proteus vulgaris* بنسبه (35.3%) عند استخدام الجين التشخيصي *Urease C*. تم التحري عن قابلية البكتريا لإنتاج انزيم ال chondroitinase واظهرت النتائج (80%) 24 عزلة من ال*P. vulgaris* منتجة للأنزيم . وتم انتخاب العزلة (p17) الاكفأ بالإنتاج ذات فعالية انزيمية بلغت (175وحدة /مل) . نقي الانزيم بثلاث خطوات قصيرة تضمنت الترسيب بأملاح كبريتات الامونيوم بنسبة اشباع بلغت 60% و الديلزة ومرر الراشح بعمود التنقية Sephadex G−150 وكانت الفعالية الانزيمية (270 وحدة/مل) وبفعالية نوعية بلغت اقصاها (692.3 وحدة /مليغرام). ازدادت الفعالية الانزيمية للأنزيم المنقى معنويا (P<0.01) اذ بلغت (563 وحدة /مل) بعد معاملة الانزيم بالحامض الاميني (التايروسين) عند التركيز(1 مولاري) بينما اظهر حامضي الاسبارتك و الكلوتامك تأثيرا مثبطا للفعالية الانزيمية بلغت ادناها (130.2وحدة/مل) و(147.3وحدة/مل) على التوالي. كذلك ازدادت الفعالية الانزيمية معنويا (P<0.01)الى (633 وحدة/مل) و(610.3 وحدة /مل) بعد المعاملة بفيتامين K و العقار بايروكسكام بتركيز(50%) على التوالي. هدفت هذه الدراسة بيان تأثير بعض الاحماض الامينية والفيتامينات وبعض العقاقير المسكنة للآلام بمختلف التراكيز على الفعالية الانزيمية.

Introduction

The genus of *Proteus* are widespread in the environment and considered as part of the microbiota in intestinal tract for humans and animals, *Proteus spp.* have been opportunistic and it,s uropathogen causing urinary tract infection in patients and in those with urinary tract abnormalities, it may lead to pyelonephritis or stones or bacteremia [1,2]. *Proteus spp.* Caused different infections in different organ such as respiratory tract, wounds, skin burns, eyes, nose , ears, throat and gastroenteritis resulting from eating contaminated meat or water or other food [3]. *Proteus vulgaris* has many virulence agents including flagella ,fimbriae, lipopolysaccharide, outer membrane proteins, capsule antigen, immunoglobulin and several enzymes such as urease, amino acid deaminases, proteases and hemolysins. The most characteristion of *Proteus*, is swarming phenomenon that enabling them to colonize and still grown in higher host [4, 5]. *P.vulgaris* has capable to produces Chondroitinase ABCI to degradation of galactosaminoglycan substrates , include DS (dermatan sulphate) ,CS (chondroitin sulphate) and hyaluronic acid [6]. The biological role of Chondroitinase was demonstrated in different studies that have mention use this enzyme in nerve regeneration following spinal cord injury [7]. Also the results of these studies were support use cABC in cartilage tissue engineering for increasing tensile properties [8]. ChABC can also stimulate plasticity of injured within spinal cord following SCI and submite a mechanism for its effects on recovery role **,** is capable of digesting molecules within scar tissue to allow some nerve fiberes to regrow [9].

Materials and Methods

Isolation and Identification

The samples of (urine, blood and ear swab) from patients cultured directely by streaking on MacConkey agar and blood agar and then incubated in aerobically conditions overnight at 37 ºC. The diagnosis of *Proteus* was achieved according to their morphological properties on macConkey agar medium, on Blood agar, Oxidase production, Catalase production, Methyl red test , Indole production, Urease production, Vogeus Proskauer tests, Kligler iron agar and Citrate utilization. The analytical Profile Index (API 20) system and molecular test by detection *Urease C* were employer for Re-conformation of the isolates. DNA extracted and purified carry out using wizard genomic DNA purification kit protocol supply by (Promega, USA). The specific primers used in this study for *Urease C* were synthesized also by (Promega, USA).The forward primer sequence is 5'CGCTTTGCGATGGCA AGTACAAGTAAG3', and that of reverse is 5'GCAAATTGAGTGACTTTGGCTGGACC3', with (263bp) as product length. The PCR program include 30 cycles were carried out: denaturation at 95 °C for 30 sec, annealing at 62 °C for 30 sec, and extension at 72 °Cfor 60 sec., after last run, the tubes were incubated at 72 °C for 7min.

Chondroitinase production

The highest isolate of chondritinas activity was transported to production media and incubated for 48 hrs at 37 ℃, Cooling centrifugation at 4 ˚C for 10 min with 9000 r.p.m, the harvested cells was washed with normal saline for 3times and suspended with 0.05 Tris Hcl buffer, pH 8.0 , then applied

Ultra-Sonication with 19600 MHz for (8cycle) (30 sec on and 90 sec off), Cooling centrifuge at 4 °C for 10 min with 9000 r.p.m. The activity of enzyme was measured according to Yamagata *et al.*, method [10].

Determination of optimal condition for chondroitinase production

-Effect of incubation period

One hundred ml of the production medium was inoculated with 10 ml of overnight bacterial suspension and incubated at 37 °C for three different times (24, 48, 72 and 96 hrs) and the chondroitinase activity was assayed [10].

-Effect of temperature

Ten ml of fresh bacterial suspension was transported to 100 ml of the production medium and incubated at different temperatures (20, 30, 37, 40, 50, 60 °C) for 48 hrs and the chondroitinase activity was assayed [10].

-Effect of pH

One hundred ml of the production medium with different pH values (4-10) were inoculated with 10 ml of activated bacterial suspension then incubated at 37°C for 48hrs, the chondroitinase activity was assayed [10].

Purification of chondroitinase

The crud chondroitinase was precipitated with 60% saturation of $(NH_4)_2SO_4$ at cooled conditions, the crystal was add gradually with continuous stirring until dissolving in ice path and centrifugation at 9000 r.p.m for10 minutes then the precipitation was dissolved gently in small volum of Tris HCl buffer solution. The solution obtained from the precipitation step was dialyzed against 0.05M, Tris HCl pH 8.0 at 4 °C for 24 - 48 hrs with stirring the volume and then the enzymatic activity and protein concentration was measured. Sephadex G-150 gel matrix was prepared according to the instructions of the manufacturer company. It was washed and suspended in 0.05M of Tris HCl pH 8.0, degassed using vacuum pump and poured in column 100×2 cm with carefully to avoid bubbles and then equilibrated with same buffer. The dialyzed enzyme was applied gently to the column, and eluted with Tris HCl buffer pH 8.0.

Protein determination

Protein concentration was estimated following the procedure of [11]. Briefly, the Protein was precipitated with 3 ml of 5 % TCA for 2ml of crude enzyme , centrifuged with 3000 r.p.m for 15 minute, and then the precipitate was dissolved with 3 ml of 0.05M NaOH solution and measure the absorbance of mixture at 235 and 280 nm. Protein concentration (mg/ml) = (O.D at 235nm- O.D at 280 nm) /2.51.

Effect of some amino acids , vitamins and NSAIDs on activity of Chondroitinase

The first step for this test is prepare different concentrations of some compounds include (amino acids, vitamins and NSAIDs drug) followed the next steps **:** add 60 µl of purified chondroitinase enzyme and add 60 µl of each compound and add 100 µl of buffer and mixed well, then incubated at a temperature 37 °C for 2 hrs , after incubation must add 140 µl of reaction solution and then re-incubated for 20 min at 37 °C, finely the enzymatic activity was assayed.

Results and Discussion

Isolation and Identification of *Proteus*

The results were mention 85 (40%) isolates were identified as *Proteus spp*. whereas 125 (60 %) samples were belonged to other bacteria. Out of 85 isolates of *Proteus* , 55 (64.7%) isolates were diagnosed as *P.mirabilis* while 30 (35.3%) isolates were belong to *P. vulgaris* by detection specific *Urease C* gene . In this study the primers used to reach rapid and accurate diagnosis of *P. vulgaris* bacteria and the result were excellent with first primer Urease C 263 bp [12]. Which give positive result for all urine samples Figure-1. Our result compatible with AL-Saadi *et al*., [13], who use species – specific primers for this gene to detect *P. vulgaris*. Molecular techniques has over convential methods is that it can provide result in 24hs` whereas routine culture followed by biochemical tests need 36-48hs.

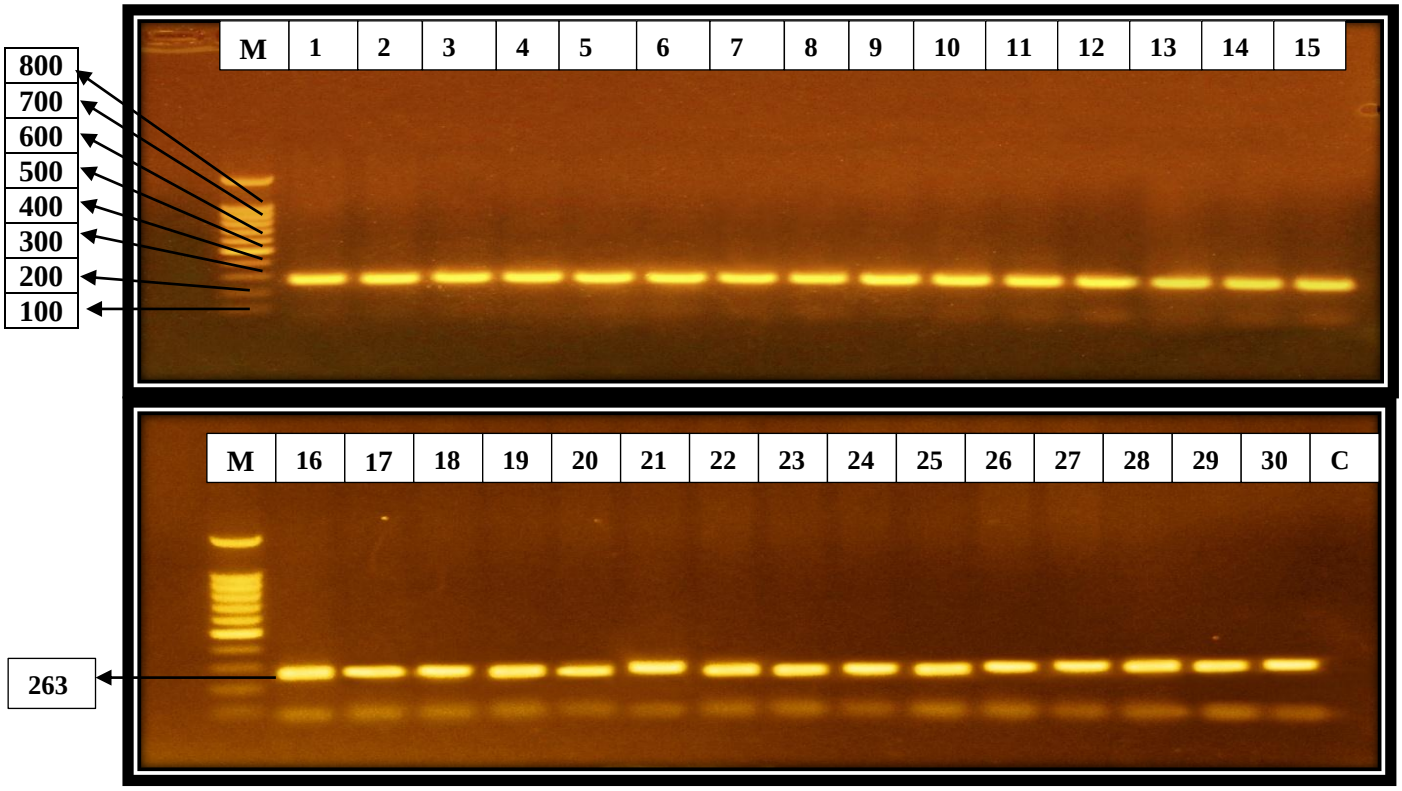

Figure 1 - Gel electrophoresis of amplified Urease C gene (263bp) of *P. vulgaris* isolates on agarose (2%), TBE buffer (1x), 75 volt for 1 hrs. stained with ethidium bromide. M: denotes DNA ladder (100 bp); Lanes 1-30 were positive. C denotes the negative control.

Investigation for chondroitinase production

The result show 24 *P.vulgaris* isolates were able to produce chondroitinase enzyme , highest activity was reported from isolates p17 (175.2U/ml), whereas isolate p9 was recoreded lowest enzymatic activity (76.8U/ml) Table-1. Isolate p17 was used to complete this study.

Table 1 - The enzyme activity of chondroitinase for isolates (substrate chondroitin sulfate C).

Number of isolates	Enzyme activity U/ml	Number of isolates	Enzyme activity U/ml	Number of isolates	Enzyme activity U/ml
1	116.2 U/ml	**11**	98.2 U/ml	**20**	112.2 U/ml
3	125.3 U/ml	**12**	92.7 U/ml	**21**	98.7 U/ml
5	84.5 U/ml	**13**	114.2 U/ml	**22**	112.6 U/ml
6	105 U/ml	**14**	117.1 U/ml	**23**	134.2 U/ml
7	134.7 U/ml	**15**	77.3 U/ml	**25**	148.2 U/ml
8	119.2 U/ml	**16**	142.2 U/ml	**27**	144.7 U/ml
9	76.8 U/ml	**17**	175.2 U/ml	**29**	115.7 U/ml
10	141.2 U/ml	**19**	149.6 U/ml	**30**	99.5 U/ml

Optimization for chondroitinase production

Effect of incubation period on chondroitinase production

The effect of incubation time (24, 48, 72,96 and 120 hrs) on the chondroitinase production was showed that the best duration period was 48 hours at (37°C), as it gave the highest activity for the enzyme peaked to (150 U/ ml) Figure-2. Our result was compatible with Abdul-Gani observation , [14], who show optimum incubation period at 48 hr. Decreasing in enzymatic activity when incubation for long period may attributed to internal digest of enzyme or change in culture conditions that affect on enzyme. Many studies show production of enzyme was increased in logarithmic phase**.**

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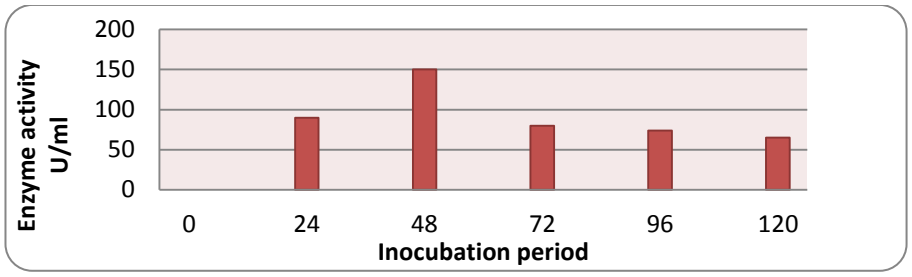

Figure 2- Effect of incubation period on chondroitinase production.

Effect of temperature on chondroitinase production

Temperatures ranged from (20 -60 °C) was applied in the production of chondroitinase enzyme, the result show the best temperature for high productivity achieved at 37 °C is155 unit/mg Figure-3. Our result compatible with Abdul-Gani and Vikas *et al*.,[14,15], who show the optimum temperature for the production of chondroitinase at 37 °C, decreasing of enzyme activity when incubated at a temperature higher than 40 °C may attributed to effect of temperatures on structure of the protein which may lead to denaturation of protein.

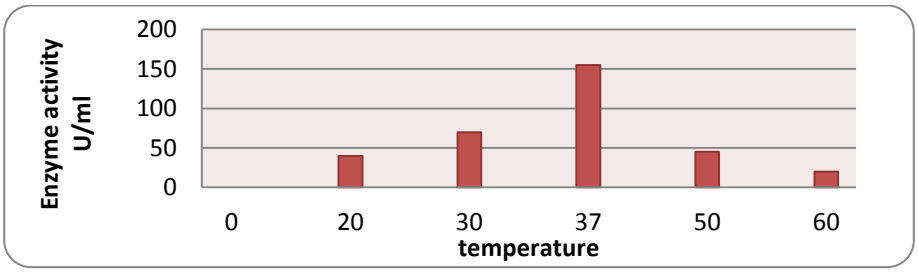

Figure 3- Effect of temperature on chondroitinase production.

Effect of pH value on chondroitinase production

The results were shown the suitable pH value for chondroitinase production was 8 with highest enzyme activity reached to (154 unit/ml), Figure- 4. This result was compitable with Abdul-Gani and Vikas *et al*., results [14,15]. In general the pH affects on the production of enzymes because it may has impact factor in the solubility of nutrients in the medium and it effect on the ionic strength in the nutrients that needed from microorganisms as well as its effect on the production and stability of enzymes and the growth of bacteria [16]. Raise of enzyme activity in pH (8) may attributed to grow of bacteria is well in slightly alkaloid media , pH value affect on characterization of media such as ionization of nutrients.

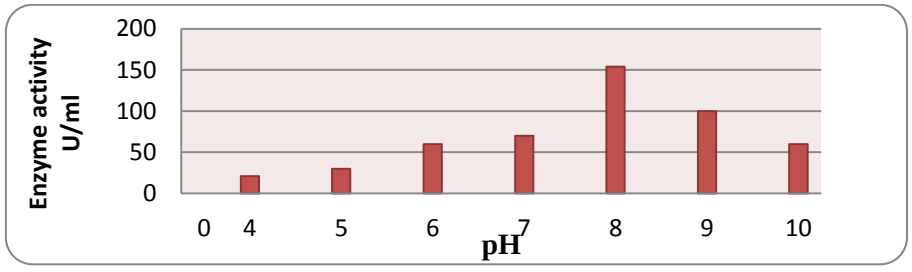

Figure 4- Effect of pH on chondroitinase enzyme production.

Chondroitinase purification

Precipitation by ammonium sulfate

Ammonium sulfate is a common salt using in protein concentration in view of higher solubility, stabilizes protein structure and cheap. The crude chondritinase was precipitate with highest amount of

protein when treated with at (60 %) saturation of ammonium sulfate .The enzyme activity and specific activity were 250 (U /ml) and 277.7(unit/mg) respectively Table- 2. This step was consider benifet in reducing the volume, which facilitated working with it. Abdul-Gani, [14] and Vasile and his co-worker [17] used the same method in In their study , they also used 60% of ammonium sulfate to obtain a higher concentration of protein,. The precipitated of Chondroitinase was dialysis against phosphate buffer for (1day);The enzyme activity was dropped slightly after dialysis , it was 240 (U/ml) with specific activity was 428.5 (unit/mg), Table-3. The dialysis membrane act as an inert sieve with a certain rate of pore size, during dialysis action water enters the dialysis bag due to the osmotic pressure of protein solution; It was very important step to remove of salts and other contaminants that may be found in the crude enzyme [18].

Gel filtration chromatography

Gel filtration chromatography by Sephadex G-150 colum (100×2cm) was the next step for chondroitinase purification produced from *P.vulgaris*. The result of eluted of filtration for purified chondroitinase was showed four peaks and the enzymatic activity was appeared in fourth peak with fractions (64-68), the specific activity was (692.3U/mg) with 72.3 % yield and six fold of purification Table- 2 and Figure- 5.

Table 2- Purification steps of chondroitinase from *P. vulgaris.*

Purifaction step	Volume (ml)	Enzyme activity (U/ml)	Protein concetration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (folds)	Yield (%)
Crude enzyme	80	70	0.6	116.6	5600	1	100
Ammonium sulfate Precipitation (60%)	7	250	0.9	277.7	1750	2.3	31
Dialysis	10	240	0.56	428.5	2400	3.6	42.8
Gel filteration by sephadex G-150	15	270	0.39	692.3	4050	6	72.3

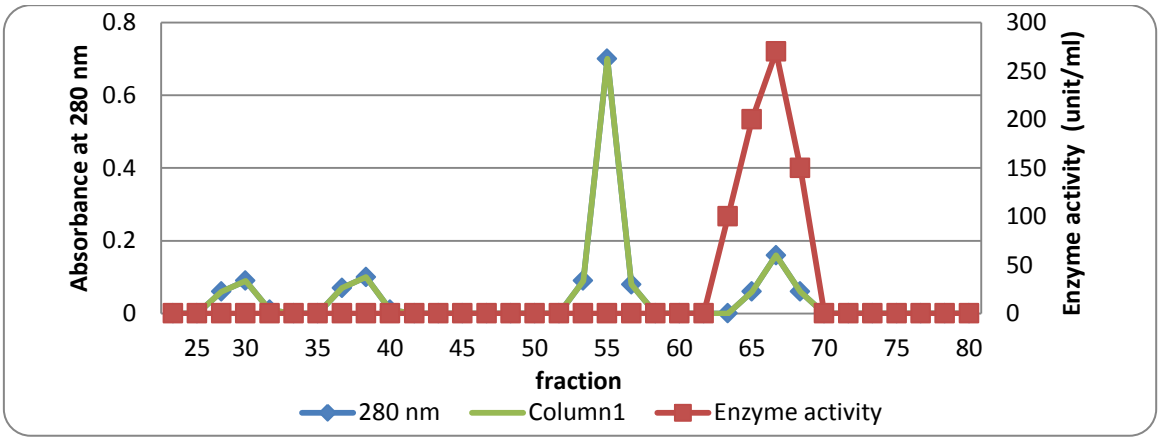

Figure 5 - Gel filtration chromatography of chondroitinase produced from *P.vulgaris* by Sephadex G-150 column (2 × 100 cm), fraction volume was (5) ml at flow rate 30 ml / hr

Effect some of amino acids on chondroitinase activity

In this study the results were shown the stimulatraly effect of Tyrosine, Arginine, Lysine, Thrionine, Glycine, Cystine , Glutamine and Aspargine on chondroitinase and the enzyme activity was raised significantly ($P<0.01$) when treated with increased of concentration gradually . whereas both Glutamic acid and Aspartic acid were shown inhibitory role ($P<0.01$) on activity of enzyme significantly , while no effect was shown for serine on activity of enzyme, Figure-6. There are few

studies related with amino acid and enzyme produced from different source. Our results compatible with other studies mention that Arginine, Glutamine and Lysine has a critical role in the lyase activity of cABCI and the cACs [15]. In other research indicate, Arginine seems to play an important role in cABC I's activity [15]. In general the enzymes are globular proteins, and their linear chains of amino acids are folded to form a three-dimensional structure. In most enzymes, amino acids are directly participate in catalysis m for that reason the sequence of the amino acids specifies the structure which in turn determines the catalytic activity of the enzyme. [19,20].

The Inhibitory role for both Aspartic acid and Glutamic acid on stability of chondroitinase enzyme , may attributed to change downward of pH value from the optimal pH (8) to acidity by effect (H atom) of these amino acids, this explain agree with Hashimoto, [21], and his co-worker opinion when they focused on kept the enzyme in acid pH , and the reduce of enzyme stability at acid pH may attributed to effect of ionic strength on structure of enzyme lead to change active site and loose it's activity[22], or perhaps in stabilizing the emulate intermediate through its positive charge [7]. Deficiency some of essential amino acid residue may cause osteoporosis, especially in the case of older women, as proven in a study from 2002. Also, study in 2003 was showed that amino acids supported the growth of osteoblasts and encouraged their division. Scientist therefore recommend that the administration of different amino acids improve all osteoporosis treatments. Amino acids stimulates production of collagen, which plays an essential function in bone and connective tissue, promotes cartilage health, skin, joint and tendon. Such as: Arginine, Glycine, Glutamine andTyrosine [22, 23].

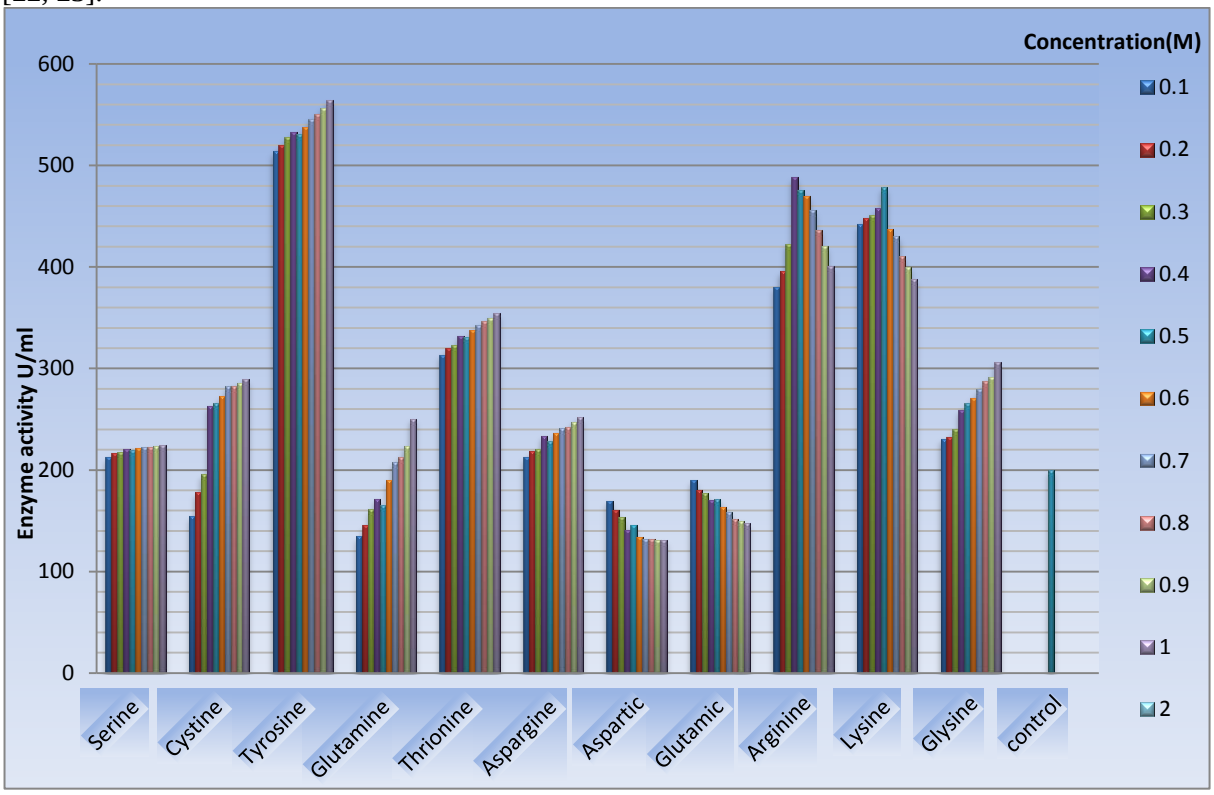

Figure 6 -Effect of amino acids: Serine, Cystine, Tyrosine, Glutamine, Thrionine, Asparagine, Aspartic, Glutamic, Arginine, Lysine and Glysine, on chondroitinase activity.

Effect vitamins on chondroitinase activity

The enzymatic activity was increased to (633.6, 389.1, 328.4, 305.8 U/ml) significantly ($P<0.01$) after treated with vitamins (K, C, E and A) respectively, while vitamin D was show inhibitory action on activity of enzyme, Figure-7. The stimulatory action of vitamins may attributed to considers as coenzymes that influence the enzyme and there are a small non protein molecules that boost the enzyme function and usually bind temporarily or permanently, and usually support the active site formation . Some coenzymes molecules like vitamins that cannot be synthesized by human

body and must be taken from the diet**,** coenzyme necessary for enzyme reaction to occur because the enzymes cannot work without coenzyme [25]. But the inhibitory style for Ascorbic acid in rising concentration may attributed to increase of acidity value and lead to loose of enzyme stability, ionic strength on structure of enzyme lead to irrversible denaturation and finally change active site and loose it's activity. This demonstration agree with Sawheney [22]. However ,vitamin C was appeared inhibitory effect on chondroitinase activity, this inhibition not coming by acidity like Ascorbic acid only ,but may attributed to different interpretations such as inefficient enzymatic interaction because the competitive behavior between vitamin and substrate about active site of enzyme that lead to inefficient enzymatic interaction and give inhibitory effect of vitamin D about chondroitinase activity. The final result for this test show the possibility of using vitamins such as vitamin (K, A, C and E) to increase the effectiveness of the chondroitinase enzyme which used to treatment of cartilage damage.

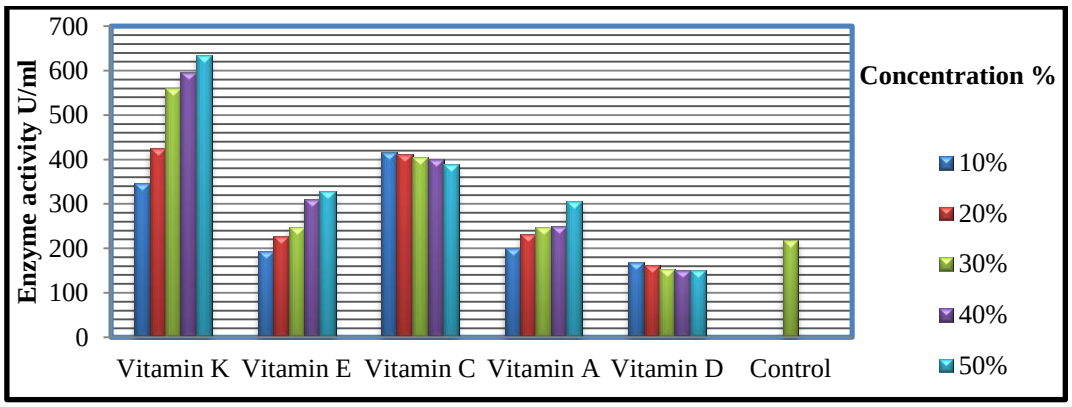

Figure7 - Effect of vitamins (K,E,C,A,D) on chondroitinase activity.

Effect some of NSAIDs on Chondroitinase

The results were show the chondroitinase activity was increase significantly ($P<0.01$) when treated with different concentration of some of NSAIDs such as Piroxicam, Diclofenac and Paracetamol,), Figure-8. In this study there are many explain mention why NSAIDs lead to increased of enzyme activity with significantly, one of them, NSAIDs may attached with amino acid in active site of enzyme and then increased affinity with their substrate (chondroitin sulfate), the second explain may attributed to effect some of ions in structural composition of NSAIDs, such as sulfur atoms that found in piroxicam, or calcium and sodium that found in structure of diclofenac, these ions may lead to attached with active site of enzyme and lead to increased enzymatic activity. There is no literatures at present time about effect of NSAIDs on chondroitinase enzyme, but our result and explain agree with Abdul-Gani and Prabhakar *et al*.,[14,26]. Prabhakar *et al*., [25] whose found that calcium preferentially increases the activity of chondroitinase ABC I toward dermatan versus chondroitin substrates in methods dependent on concentration. Calcium attached to chondroitinase ABC I through specific residue of amino acids that could play article role in calcium coordination. Abdul-Gani, [13] in his report showed that $Cacl_2$ and $Mgcl_2$ gave the highest remain activity of chondroitinase . From this study may conclude that chance of increasing enzyme activity when synergized with amino acid, vitamins and NSAIDs to use it in medical purpose.

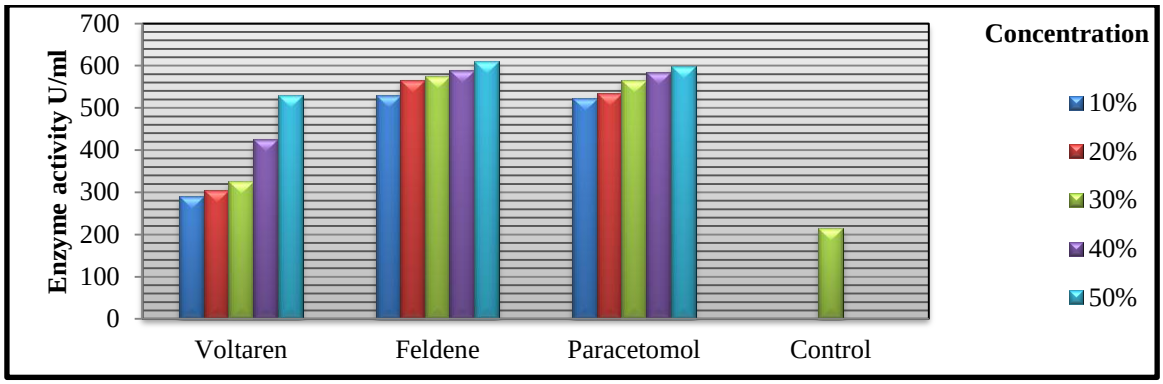

Figure 8- Effect some of pharmaceutical drugs suh as Voltaren, Feldene and Paracetomol on chondroitinase activity

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