Partial purification of Hyaluronate lyase from *Streptococcus pneumoniae*, and study the effect of some materials as inhibitors on it

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Abstract:
The study included investigating *Streptococcus pneumoniae*to secrete the Hyaluronate lyase as spreading factor qualitatively and quantitatively, enzyme purification methods by(Ammonium sulfate precipitation 80%, Dialysis, Gel-filtration G150 and Ion-exchange chromatography) and investigating of the inhibitory activity of some chemicals towards the activity of the enzyme.It was noted that all isolates were Hylase producers, but in varying proportions when grown in certain conditions,such as (BHI broth supplemented with HA substrate 0.4 $mg.mL^{-1}$), pH 7.2±2, 35°C for 18-24 hours an incubation period of with continuous stirring of the samples. The production was varying proportions according to serotype. Agarose medium supplemented with HA substrate (0.4 $mg.mL^{-1}$) presence phenol red (0.025 $g.L^{-1}$) was also tested and developed to investigate Hylase enzyme production from bacteria inoculated in the medium. The specific activity in crude of enzyme was (10 U/mg). The specific activity of the enzyme increased from 20 U/mg to 150 U/mg and by 70% of the enzyme yield upon gel filtration.

Using the Schrödinger Maestro programversion (12.5.139), a preliminary test (simulation) was conducted for the selected materials to verify the amount of their adhesion to the enzyme and thus to know their activity by inhibiting the enzyme, then their inhibitory activity was verified in the laboratory, included (L-ascorbic acid, nicotinic acid, acetyl salicylic acid, folic acid and 4-aminobenzoic acid). It was found that L-ascorbic acid gave the highest inhibition at a concentration of (0.1)M without dilution, as its remaining activity reached to 57.2%. While, the percentage of remaining activity of 4-aminobenzoic acid at a concentration of (0.1)M was (70%) in the laboratory, but when tested by the program, it gave a docking score of -0.5926 $kcal.mol^{-1}$, although there were other substances that gave a higher docking value than it. As for the other substances, at a concentration of (0.1)M it did not give a high inhibition activity, but by increasing the concentration it gave inhibition.

Keywords: *Streptococcus pneumoniae*, Hyaluronate lyase, Agarose-phenol red medium, L-ascorbic acid, 4-aminobenzoic acid, Schrödinger Maestro program.

1. Introduction

Streptococcus pneumoniae has many virulence factors that enable it to cause infections and penetrate deep places in the body, and among these factors is the enzyme Hyaluronate lyase (Hylase)or is known as (spreading factor) (El-Safory *et al*., 2010). This enzyme acts on breaks down hyaluronic acid, a polymer of nonsulfated linear polysaccharide which is a component of connective tissue, acts on lubricants in joints also works to give softness to tissues besides collagen-by elimination of β(1-4) bond between N-acetyl-D-glucosamine(←1-β-4→)D-glucuronic acid, releasing unsaturated disaccharides (Hynes & Walton, 2000). Many studies have been conducted on it,

including its description and purification, due to its important role in pathogenesis, as well as in the pharmaceutical industries (Jung, 2020).

2. Materials and Methods

2.1- Samples collection

(200) different samples were collected from children suspected of having pneumococcal infection who showed symptoms of infection, and the ages of the infected were distributed between (a few days to 14 years) of both sexes included (145) samples taken from males and (55) samples from females, Samples were collected from children's hospitals located on both sides of the city of Mosul, such as (Mosul teaching hospital, Ibn Al-Atheer teaching hospital, Al-Salam teaching hospital, Al-Faisaliah medical center for chest diseases), during the period from November 2021 to April 2022. The samples included (nasopharyngeal (NP) swabs, acute otitis media (AOM) swabs, sputum, blood and CSF).

2.2- Isolation of *S.pneumoniae*

The specimens were inoculated on blood agar & Brain-heart infusion broth. BA plates were incubated in 37 $^{\circ}$C, presence CO_2 (5-10%) with inverted place for 18-24 h. Then, colonies suspicious of alpha-hemolysis and typical morphology (color, edges, glistening and mucous) were treated with Gram’s stain, catalase and sub-cultured on blood agar plates with optochindiscs incubated again. The optochin sensitive isolates were sub-cultured to obtain pure growth.

2.3- Agarose-phenol red plate assay

It is an innovative prepared solid medium used to detect the Hylase enzyme production by bacteria (e.g. *S.pneumoniae*) as a qualitative assay. It was prepared by adding (0.04 g) aqueous solution of sterile HA sodium to autoclaved (100 mL) of agarose (0.9%, w/v) with aphenol red (0.0025 g. 100 mL^{-1}) as a pH indicator, while the Gram’s iodine-plate assay is not contain phenol red in its composition (Patil and Chaudhari, 2017). Mixed well to give final concentration (0.4 $mg.mL^{-1}$) of HA substrate, and adjust the pH of the medium to 7.2±0.2 and the medium was poured to a depth of 3-4 mm. After solidification, 3 to 4 wells of (5 mm) in diameter were prepared by sterile cork borer. Plates were stored at 4 $^{\circ}$C to provide a firm surface and in an inverted position, see (fig 2-1).

Figure (2-1): Showing an agarose-phenol plate in presence hyaluronic acid for detection of Hylase production

Table (2-1): The contents of agarose-phenol red medium per (100 mL), pH 7.2±0.2

No.	Material	Amount
	Agarose	0.9 g
	Hyaluronic acid substrate	0.04 g
	Phenol red	0.0025 g

2.4- Identification of *S.pneumoniae*

Samples examined by microscopic examination to examine the bacteria from the suspected colonies under microscope after dyeing it by Gram's stain to see the (pneumococcus cell shape and size), catalase test, morphological identification of colonies characteristics including (color, shape, size, edges, consistency),optochin sensitivity test, bile solubility test and more confirmatory test such as (agglutination latex test used Pastorex® meningitis kit and VITEK 2 system to identification of biochemical tests).

2.5- Prepare the cell-free filtrate supernatant (CFFS)

Three pneumococcal isolates selected of the total isolates to detect their Hylase productivity from clinical samples based on (α-hemolysis, optochin sensitivity colony size and shape):

1- (Spn7) from nasal of cerebral palsy patients (CP) infected pneumonia.
2- (Spn10) from sputum of (Down syndrome) patient have chest infection.
3- (Spn17) isolate from clinical AOM.

Sub-cultured these isolates on BA at 37 °C, 24 h presence CO_2 5-10%(in anaerobic jar). After this, scraped pneumococci of previous culture and transferred into (100 mL) of sterile BHI broth for each separately and supplemented with HA substrate (60 mg, 0.6 $mg.mL^{-1}$) to induced production, and incubated in 37 °C for 18 h with aerobic conditions on rotary shaker. Then, (5 mL) of culture broth withdrawn and centrifuged at 7513 ×g for 20 min at 4 °C by cooling centrifuge. The clear supernatant collected wassubsequently used for assay and purification (Tam and Chan, 1985).

2.6- Determination of SpnHl activity

The enzymatic activity assay is based on turbidimetric method prepared by Kass (Kass and Seastone, 1944), and modified by Dorfman (Dorfman and Ott, 1948). After reactivating the three pneumococcal isolates and inoculating them in 100 mL of BHI broth supplemented with hyaluronic acid, taking (0.5 mL, 0.07 $mg.mL^{-1}$) of HA substrate and added to (0.5 mL) of CFFS (crude enzyme) with (0.5 mL) of [0.05] M sodium phosphate buffer and in the presence of [0.05] M NaCl at pH 7.0 and they were incubated at 37 °C for 30 minutes in a water bath. Then, (0.5 mL) of the mixture was withdrawn and mixed with (2.5 mL) of acidified albumin dissolved in sodium acetate solution ([0.5] M, pH 3.1), then the reaction was stopped by adding (0.5 mL) of TCA, there after incubate the solution for 10 minutes in a water bath at 37 °C. The blank was prepared without CFFS added to the mixture (Tam and Chan, 1985). The turbidimetric assay for Hylase belonging to group EC (4.2.2.1) produced from bacteria (e.g., *S.pneumoniae*) was measured by a spectrophotometer (UV-VIS, Bio-Rad) at a wavelength 600 nm. One unit of Hylase activity was defined as the amount of enzyme required to reduce the resulting turbidity to (half the amount of substrate added) in 30 minutes and under specified conditions. (Hill, 1976). SpnHl activity was measured during all partial characterization stages.

2.7- Determination of protein concentration

Determination of protein concentration according to Bradford method (Bradford, 1976) which depends on binding the coomassie brilliant blue G-250 dye with the protein at room temperature and forming a dye complex, then measured absorbance at (595 nm).Take (0.05 mL) of

the sample and add (2.5 mL) of Coomassie brilliant blue G-250 dye and (0.45 mL) of phosphate buffer [0.05]M in was added, mix by inversion and incubate at room temperature for 2 min. Then, measure the absorbance at wavelength (595 nm) for all samples. The blank was prepared from (2.5 mL) of Coomassie dye and (0.45 mL) of phosphate buffer to reset spectrophotometer. A standard curve was plotted between the BSA concentrations against the corresponding absorbance at (595 nm).

2.8- Partial purification of SpnHl

The isolate (Spn7) was selected for enzyme purification methods because of high productivity and activity of Hylase enzyme. The Hylase purification methods were carried out according to (Jain, Jain and Jain, 2020)

2.8.1- Precipitation by using ammonium sulfate

This process is the first stage of purification, during which the protein present in the liquid is precipitated depending on the saturation ratio of ammonium sulfate in the solution. Precipitation is done with ammonium sulfate by gradually adding ammonium sulfate whereby (28.05 g) of ammonium sulfate was dissolved in (50 mL) of D.W at a saturation rate (80%). (40 mL) of the crude supernatant (crude enzyme) was dissolved in it and mixed gently on a magnetic stirrer for 2 h at 4 ˚C (in an ice beaker). Then, it was centrifuged at 4226 ×g for 20 minutes at 4 ˚C, taking the precipitate proteins and dissolved it in (50 mL) of phosphate buffer at pH 7.0 and neglect supernatant. Then, the enzyme activity and protein determination was estimated.

2.8.2- Sort (crude enzyme) using dialysis bags

After precipitation with ammonium sulfate, further purification steps are performed on the sample to concentrate the Hylase enzyme, such as dialysis. At first, dialysis membranes tubes (7 KDa.) MWCO size are placed in water bath at 60 ˚C for 10 minutes to activated it and become more easy to open. Then, open each tube individually and one end of the tube is closed with a tight thread and put (40 mL) of the precipitate obtained from ammonium sulfate, after which, close the other end tightly and then the bag is placed in phosphate buffer solution pH 7.0 with continuous stirring using a magnetic stirrer for 24 h, with the buffer solution changed every 3 h to remove sulfate ions. Also, dialysis bags placed in sucrose are left for 2 h until their volume is about half or less. The dialysis separation process was carried out at 4 ˚C to maintain enzyme activity. Next, take the filtrate (20 mL) from the dialysis bag into an ion exchange separation column.

2.8.3- Ion-exchange chromatography (2x25cm)

DEAE-cellulose column was prepared according to (Whitaker and Bernhard, 1972) by suspense (20 g) of resin in (1 L) of D.W. then the mixture was left for 24 h to settle down, removed the supernatant and rewashed several times with D.W until getting clear appearance. The suspension was filtered throughout Whatman No.1 using buchner funnel then degased by vacuum pump. The resin was activated in [0.25] M NaCl for 30 minutes and wash it with D.W. The suspension was filtered again as mentioned above and washed several times with [0.25]M of HCl buffer and next by D.W before it was equilibrated with phosphate buffer [0.05]M, pH 7.0. Repeated washing with solutions of different molar concentrations is intended to rotate the charge of the column.After preparation the column, the protein (crude enzyme) solution obtained from gel filtration step (20 mL) was then added slowly on top of the surface to IEX column (2x25cm) equilibrated previously with phosphate bufferand the solution is left inside the column for (3-5) minutes to soak into the column. Separation is done by using (25 mL) of phosphate buffer, and (40) tubes of excluded fractions are collected with a volume of (2 mL) for each part. Then, the column was washed with an equal volume of phosphate buffer, while the bound proteins were eluted with five molar gradient concentrations of NaCl [0.1, 0.3, 0.5, 0.7, 0.9 to 1.0]M respectively. Flow rate throughout the column was (30 $mL.h^{-1}$) and the absorbance of each fraction was measured at (280 nm) using UV-VIS spectrophotometer.

2.8.4- Gel-filtration chromatography (2.5×35 cm)

Gel-filtration column was prepared as recommended by (Pharmacia fine chemicals) company. A (5 g) of Sephadex G-150 was suspended in (1 L) of [0.05]M phosphate buffer pH 7.0, then heat it up to 90 °C for 5 h to ensure the swelling of the beads with stir the gel gently with a sterile glass stick from time to time. Next, transfer the gel to a graduated cylinder and the solution is left for 20 minutes to cool down,then remove the top part of the buffer and resuspend the gel in (600 mL) of the phosphate buffer and degased by vacuum pump, and pour the gel slowly in column diminished as (2.5×35 cm) to prevent formation bubbles and the column was equilibrated and washing with the same buffer that's used in gel suspended at rate flow (30 $mL.h^{-1}$) to the next day. After preparing the gel column and equilibrating it with phosphate buffer as mentioned in the previous step, (30 mL) of concentrated protein (crude enzyme) obtained after the dialysis purification step was applied onto the column. Elution was achieved at a flow rate of 3 mL/fraction, and the same buffer was used for equilibration. Absorbance of each fraction was measured at 280 nm. Hylase activity was also determined in each fraction and protein concentration was determined.

2.9- Partial characterization of SpnHl kinetics

Some of the kinetic properties of the partially purified SpnHl were determined towards (Michaelis-Menten constant, approximate molecular weight and estimation of the inhibitory effect of the selected chemical materials on Hylase activity) and their effects on enzyme activity and stability of enzyme were determined as will be described later, and the absorbance was measured at a wavelength of 600 nm by (UV-VIS, Bio Rad) with blank preparation for each step.

2.9.1- Estimation of approximate molecular weight of SpnHl by gel filtration chromatography

Molecular weight of the SpnHl was estimated by Whitaker and Bernhard (1972) by used gel filtration chromatography recommended by (Sigma-Aldrich company). Sephadex G-150 column (2x40 cm) equilibrated with [0.05]M phosphate buffer pH 7.0 was used, and elution was performed with the same buffer. Proteins of known molecular weight were used as markers, mentioned in table (2-2). The void volume ($V_{\circ}$) was estimated by blue dextran at (600 nm), and the elution volume (V_e) for each standard protein was measured at (280 nm) by using (US-VIS Bio-Rad) spectrophotometer from the elution volume of the Hylase. Then, we drew a linear relationship between the volumes of the standard proteins elution solutions and the logarithm of their known molecular weights by comparing the volume of the standard proteins elution solutions with known molecular weight and the volume of Hylase elution, then the linear relationship between $V_e/V_{\circ}$ against log molecular weight for each standard protein was plotted (Whitaker and Bernhard, 1972).

Table (2-2): Standards proteins solutions with weights and molecular weights

No.	Protein	Weight ($mg.mL^{-1}$)	Approximate molecular weight (kDa)
	Albumin	10	66
	Alcohol dehydrogenase	5	150
	Carbonic anhydrase	3	29
	Lysozyme	3	14

2.9.2- Determination the values of the Michaelis-Menten of partial purified SpnHl

To set the values of MM equation (Vmax, Km) of the SpnHl it is calculated by using different concentrations of the HA substrates (0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 2.0) $mg.mL^{-1}$, with [0.1]M of enzyme, and measuring the activity enzyme with each concentration and observing the maximum

velocity that the enzyme activity reaches which represents (Vmax), the value of Km was determined too.

2.9.3- Estimation of the inhibitory effect of the selected chemical materials on SpnHl activity

Some chemicals molecules were selected for the purpose of studying their inhibitory activity on partially purified SpnHl enzyme activity, included (acetylsalicylic acid, folic acid, L-ascorbic acid, 4-aminobenzoic acid and nicotinic acid). This selection was made after test these materials (simulating them) in Schrödinger maestro program and studying its binding with protein (enzyme). Different molar concentrations [0.1, 0.2, 0.3, 0.4, 0.5] M of each molecules were used for each.

2.10- Materials testing using Schrödinger maestro program

Initially, the selected molecules were tested for their enzyme-inhibiting activity using the Schrödinger maestro program version (12.5.139), which is one of the computer programs used in the chemical-biological research field.

2.10.1- Determination of optimal molar concentration of inhibitors

The test tubes containing (0.5 mL) of the different amounts of HA substrate (0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 2.0) $mg.mL^{-1}$ in PBS solution at pH 7.0 were incubated with (0.5 mL) of each molar concentration of the inhibitor after they were dissolved in the appropriate solvent, under sterile conditions with (0.5 mL) of partial purified SpnHl at 37 °C for 15 minutes, while the control tube was prepared without addition the inhibitor. The method mentioned in (2.6) was also used to measure the activity of Hylase for each material, and the control tube prepared with each molar concentration of inhibitor. Then the absorbance was measured at 600 nm. The inhibition percentage was calculated by the following equation (Segel, 1991).

$$\%\ \textbf{Inhibition} = \textbf{100} - \frac{\textbf{Activity without inhibitor}}{\textbf{Activity with inhibitor}} \times \textbf{100}$$

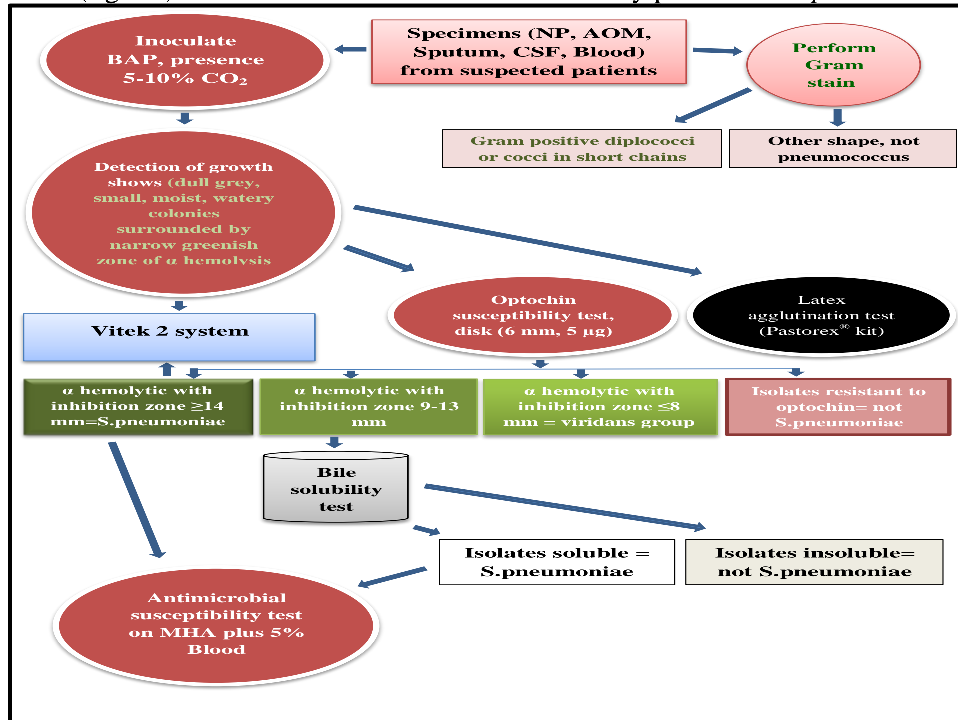
3. Results and Discussion

3.1- Isolation of *S.pneumoniae*

Two-hundred clinical samples were collected from children suspected of having pneumococcal infection based on the initial clinical diagnosis by the physician. The total number of positive infections with pneumococcal infection was (47) cases (23.5%) out of the total 200 cases, distributed among (33) males (16.5%), and (14) females (7%), see (fig 1-1). The results of body sites samples distributed between (nasal swabs 46.81%, AOM swabs 25.53%, throat swabs 14.9%, sputum 10.64%, blood 0 and CSF 2.21%) samples.

3.2- Identification of *S.pneumoniae*

The (fig 3-1) summarize the identification laboratory process for *S.pneumoniae*



3.3- Qualitative detection of SpnHl production by Agarose-Phenol red plate assay

This assay represent a wells-diffusion method. Since the bacteria are not able to consume agarose as nutrients, it was used to avoid any interference in the result.by used a tiny amount of HA substrate and agarose instead of other nutrients. Added (50 µL) of pneumococci supernatant prepared in (2.5) into the separate wells along with heat treated culture supernatant as a negative control. The plates were incubated at 37 ˚C for 18 h. After the end of the incubation, we notice the color change around the inoculated wells to yellow, and the area of hyaluronic decomposition is determined by the SpnHl enzyme.

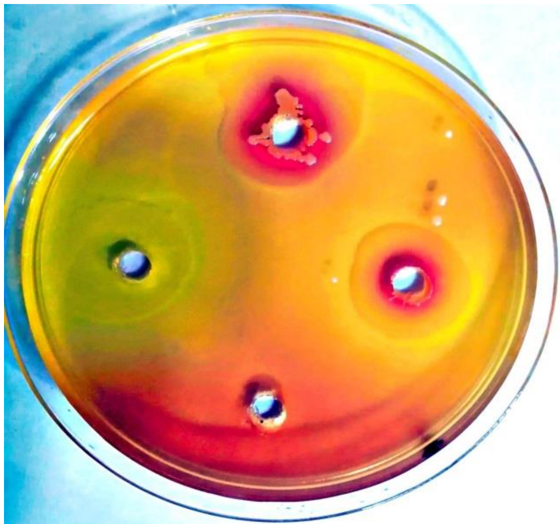


Figure (3-2): Shows an agarose-phenol red plate inoculated with the pneumococcal filtrate. Where the hole on the left and right shows a region of high enzyme yield in yellow with two diameters zones, the first zone shows the spreading of the filtrate, and the second zone represents the maximum area of hyaluronic degradation.The well at the top appears to show poor secretion. While the well below shows heated filtrate as control (no production of enzyme and no color change).

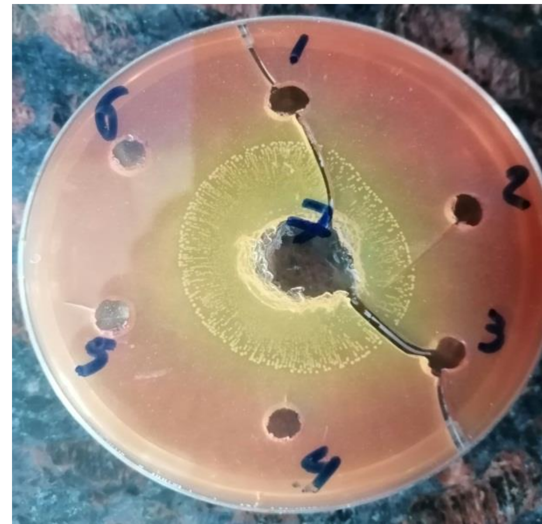


Figure (3-3): Shows an agarose-phenol red plateinoculated in the middle well and noted the production of the enzyme, while the pits on the edges are not inoculated.

3.4- Quantitative detection of SpnHl

The specific activity of the three isolates from the total isolates was tested based on the qualitative variance in the lysis diameter in secretion of the enzyme on agarose-phenol medium, where the specific activity of the enzyme recorded a variation of the isolates between (10 to 5.5 U/mg) of the crude, and the (Spn7) isolate recorded the highest value, perhaps the reason for this is due to the nature and location of the injury, as mentioned by (Hynes & Walton, 2000), and the gene expression of enzyme secretion also depends on the type and location of the injury (Asmaa, 2014; Ibrahim & Eldirdiri, 2019). See table (3-1).

Table (3-1): The specific activity and lysis diameters of selected isolates

Isolate	Specific activity of crude protein (U/mg)	Lysis zone diameter (mm)
Spn7	10	25
Spn10	8.2	16
Spn17	5.5	5

3.5- Partially purified SpnHl

Four different purification methods were used to obtain partially purified Hylase from Spn7, included:

3.5.1- Precipitation using ammonium sulfate

The results showed that this ratio gave a specific protein activity (20 U/mg), indicating a significant increase in the specific activity compared to the crude protein extract (10 U/mg), see table (3-2). The saturation and specific activity of Hylase precipitation differ from one bacterial species to another, and even among species belonging to the same enzyme group. In *Staphylococcus aureus*, Asmaa, 2014reached to the saturation (90%), while in *Propionibacterium acnes* (60 to 90%) the percentage of saturation (Ingham *et al*., 1979).

3.5.2- Sorting with dialysis bags

the results in table (4-5) of the specific activity of the enzyme increased to (34.66 U/mg), and the enzyme activity increased from 4.0 U/mL of the enzyme activity precipitated with ammonium sulfate to (5.2 U/mL) after the membrane separation process, due to the removal of other inhibitors that reduce the activity of enzyme.

3.5.3- Gel filtration chromatography

Fractions were collected from Sephadex G-150 column (2.5×35 cm) previously equilibrated with [0.05] M phosphate buffer solution pH 7.0. After conducting the measurements, the results in (fig 4-20) showed that one peak of protein concentration with one peak of Hylase activity for the fractions (15-23), and the specific activity of the enzyme was reported (150 U/mg) with (15) of purification fold and yield (70%), see table (3-2).Hamai *et al*., 1989 purified Hylase from *Streptococcus dysagalactiae* using Sephacryl S-300, with folds of purification reached 27 times.

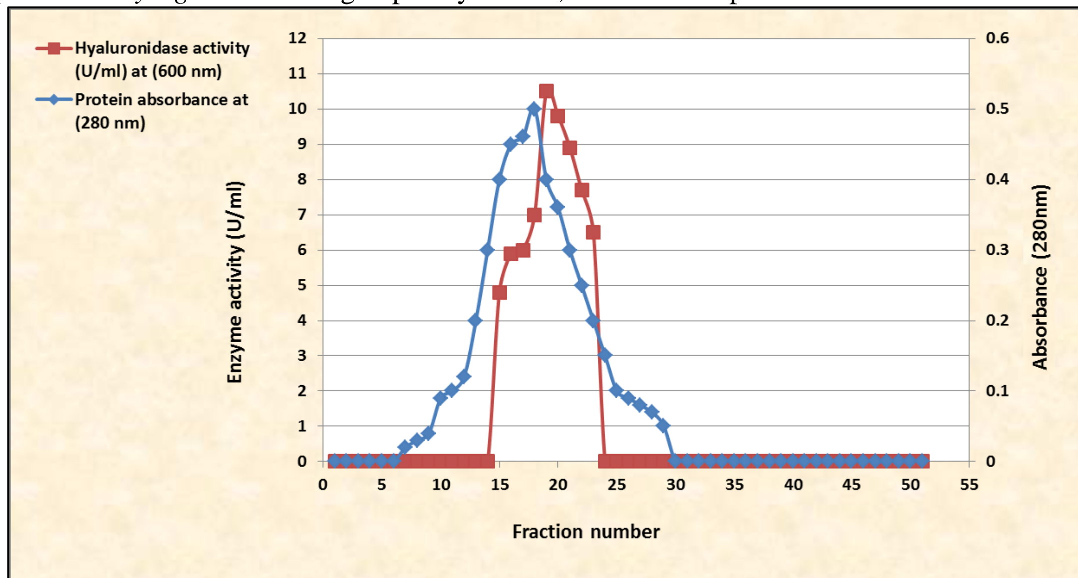


Figure (3-4): Showing the results of Hylase purification by gel-filtration from *S.pneumoniae*, using a Sephadex G150.

2.5.4- Ion-exchange chromatography

the results in (fig3-5) showed that there was one protein peak appeared after elution by the gradient concentrations of sodium chloride with one peak of enzyme activity in the washing step, and eluted proteins fractions (42 to 50) contained most of the Hylase activity when reached to (10 U/mL). Through these results, it is clear that SpnHl has a net negative charge, due to its association with the

anionic ion exchange (DEAE-cellulose). The specific activity of the enzyme was measured as (111.11 U/mg) protein, also its purification fold reached to (11.11) with (50 %) overall yield, see table (3-2).J. H. Ozegowski *et al*., 1994 used this exchanger to purify the enzyme from *Streptococcus dysagalactiae*. Asmaa, 2014 used this assay to purify the enzyme from *Staphylococcus aureus*.

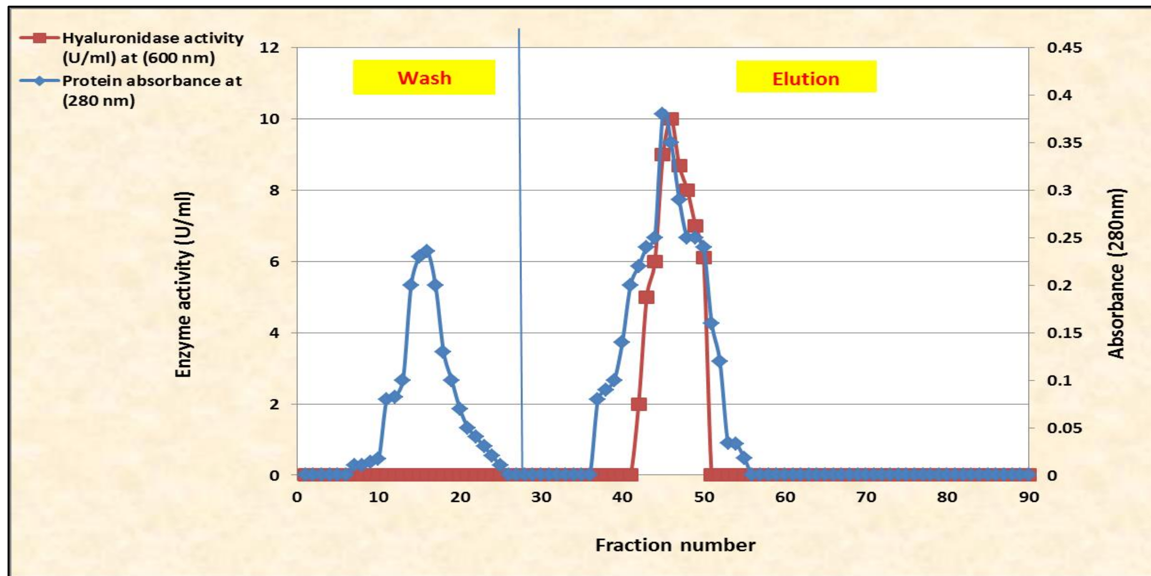


Figure (3-5): Showing the results of Hylase purification using IEX chromatography from *S.pneumoniae* using a DEAE-cellulose.

Table (3-2): The methods and results for the partial purification of *Streptococcus pneumoniae*Hyaluronate lyase (SpnHl).

Purification step	Volume (mL)	Enzyme activity (U/mL)	Protein concn (mg/mL)	Specific activity (U/mg)	Total protein (mg)	Total activity (U)	Fold of Purification	Yield (%)
Crude protein (enzyme)	100	3	0.3	10	30	300	1	100
Ammonium sulfate precipitation (80%)	40	4.0	0.2	20	8	160	2	53.33
Dialysis	30	5.2	0.15	34.66	4.5	156	3.46	52
Gel-filtration chromatography Sephadex G150	20	10.5	0.07	150	1.4	210	15	70
IEX chromatography DEAE-cellulose	15	10	0.09	111.11	1.35	150	11.11	50

3.6- Estimation of the approximate molecular weight of the SpnHl

Whitaker and Bernhard method using gel filtration chromatography technique was followed to estimate the approximate molecular weight of native hyaluronidase enzyme purified from *S.pneumoniae*(Whitaker & Bernhard, 1972). The results of the standard curve for determining the molecular weight indicate that the approximate molecular weight of the native Hylase enzyme was (110 kDa), see (fig3-6). This result was close with Jedrzejas and his colleagues reached to the native enzyme 107 kDa, also showed that the molecular weight of the Hylase enzymes varies between (107, 94, 91, and 89 kDa) although not all forms of the enzyme have been characterized (Jedrzejas *et al*., 1998). This variation range is due to several reasons including; the strain from which the enzyme was extracted, the extent of genetic transformation in the culture, the type and number of cloning vectors used, the nature of the gene sequence and gene expression for enzyme during the translation process, the method used to determine the molecular weight, and the modifications that occur to the enzyme during secretion between the outer of the cell and inside the host tissue and converting it from its native form to the active form. (Li *et al*., 2000;Ponnuraj & Jedrzejas, 2000). For example, the molecular weight of Hylase extracted from *Staphylococcus aureus* was 84 kDa (Makris *et al*., 2004), while in *Streptococcus agalactiae* was 92.111 kDa and also differ according to strain, although both species belong to the same group of enzyme (Jedrzejas & Chantalat, 2000).

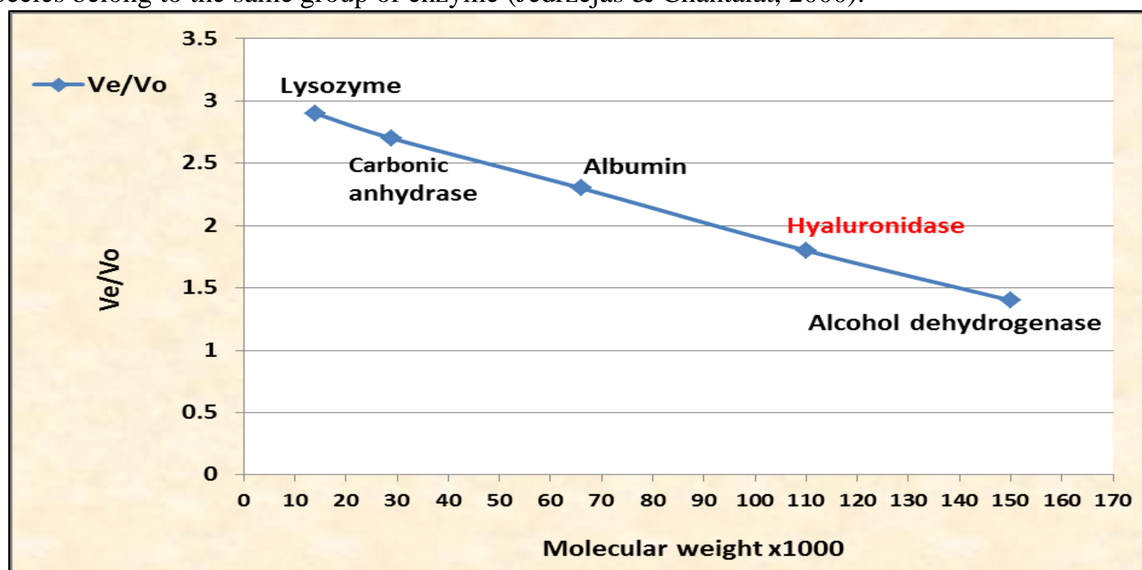


Figure (3-6): Shows the approximate molecular weight of native Hylase produced from *S.pneumoniae.*

3.7- Determine the effect of substrate concentration on reaction rate

The graph in (fig3-7) shows that the speed of the enzyme reaction began to increase with increasing concentration until the concentration (1.6 mg/mL) was reached, when the reaction speed recorded the highest value (0.47 U/mL), which represents the (Vo) of the enzyme activity. But by increasing the concentration after this value, noted that there is no increase in the rate of the enzyme activity, which indicates that the active site of the enzyme has been saturated with the HA substrate. For the purpose of estimating the type of reaction and using different concentrations of the base substrate with one concentration of the enzyme and by reversing the MM equation, we can calculate

the values of (Km,Vmax) by using the Lineweaver-Burk equation, where the value of Km=2.84 while the value of Vmax = 1.3, see (fig 3-8).

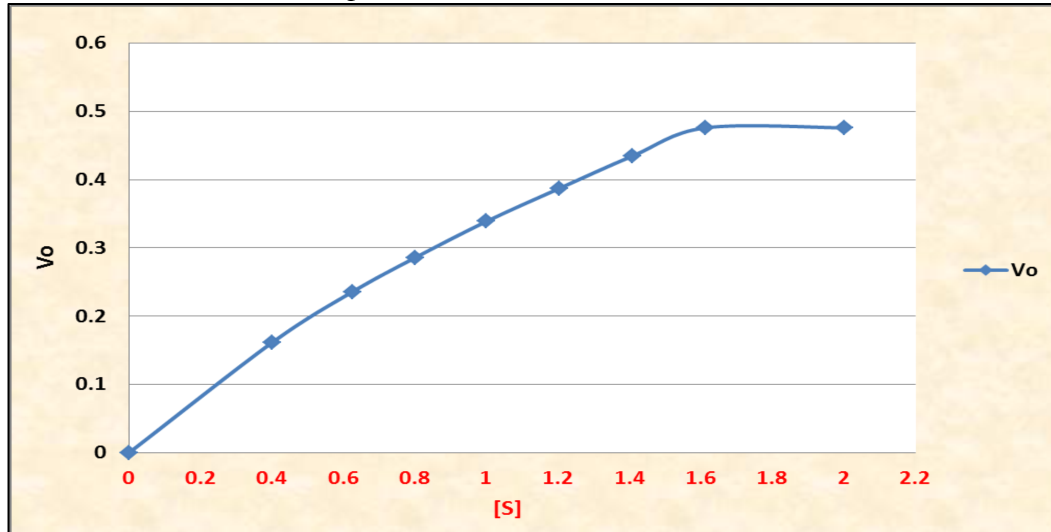


Figure (3-7): Shows the effect of substrate concentration [S] on enzyme reaction rate (Vo).

3.8- Estimation of inhibition rate

The partially purified SpnHl was incubated with the molecules (1-5) listed with different molar concentrations and the percentages of remaining activity for these molecules listed in table (3-3).

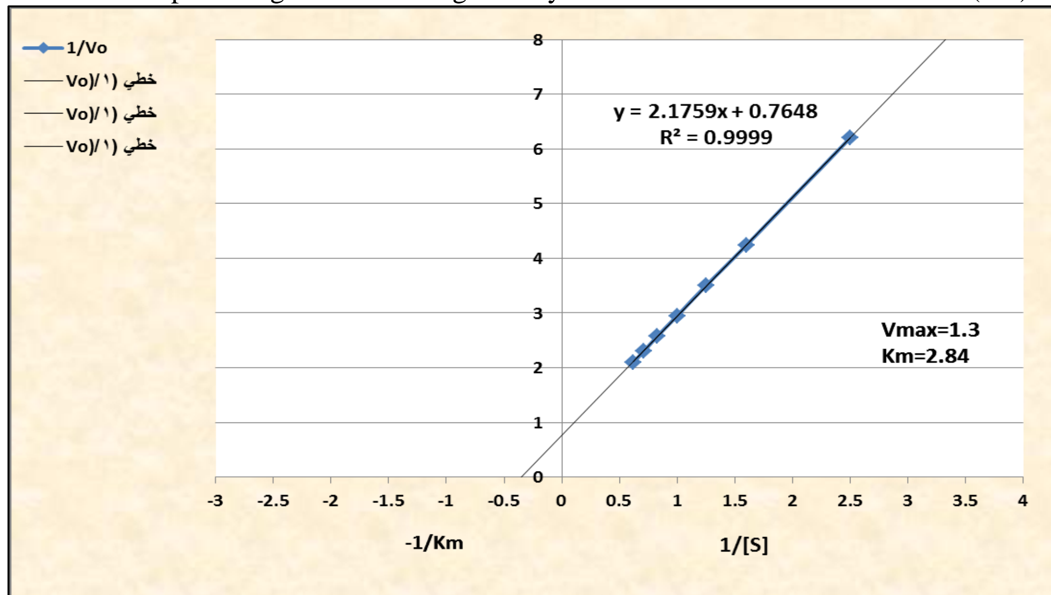


Figure (3-8): Shows Lineweaver-Burk equation represent an inverse of MM equation.

Table (3-3): The molecules used as inhibitors, their concentration, and the percentage of remaining activity.

	L-ascorbic acid (Vc)	**Solvent:** DMSO

Molar concentration [M]	Hylase activity (U/L)	% Remaining activity without dilution	% Remaining activity with dilution
0.1	2.3	57.5	28.75
0.2	2.25	56.2	28.1
0.3	2.07	50	25
0.4	1.95	48.7	24.35
0.5	1.89	47.26	23.63
	Acetyl salicylic acid (Aspirin®)		**Solvent:** DMSO
0.1	3.8	190	95
0.2	2.78	135	67.5
0.3	2.6	130	65
0.4	0.17	85	42.5
0.5	0.1	35	17.5
	Folic acid (VB_9)		**Solvent:** D.W.
0.1	3.0	150	75
0.2	1.9	95	47.5
0.3	0.7	35	17.5
0.4	0.6	30	15
0.5	0.55	27.5	13.7
	Nicotinic acid (VB_3)		**Solvent:** D.W.
0.1	4	200	100
0.2	2.5	145	72.5
0.3	2.4	120	60
0.4	2.2	110	55
0.5	2	50	25
	4-aminobenzoic acid (VB_{10})		**Solvent:** DMSO
0.1	1.46	70	35
0.2	1.2	60	30
0.3	1	50	25
0.4	0.81	40.50	20.25
0.5	0.8	40	20

From these results, it is clear that 'Vc' gave the highest inhibition of the enzyme by (57.5%) of the remaining activity at a lowest concentration of [0.1]M without dilution, and the docking score showed (-5.029 kcal.mol^{-1}) and RMS (0.03826 Å), while VB_{10} gave the highest inhibition of the enzyme by (70%) of the remaining activity at a lowest concentration of [0.1]M without dilution, and the docking score showed (-5.926 kcal.mol^{-1}) and RMS (0.03803 Å). This result is consistent with what was confirmed by Li *et al*., (2001) about that (Vc) is selective inhibitor of SpnHl, and the

hydrophobic interaction possible increased inhibitory activity.Also, It is possible that the structure of Vc plays a major role in the affinity for inhibition and interference with the base substance, being a hexose sugar derivative. This selectivity plays an important role in the possibility of using (Vc) as a specific selective therapy as an alternative to antibiotics in the treatment of pneumococcal infections (Botzki *et al*., 2004).

Also noted that 4-aminobenzoic acid (B_{10}) gave a high inhibition rate with a remaining activity of (70%) at a lowest molar concentration of [0.1]M without dilution, docking score for this molecule showed (-5.926 kcal.mol^{-1}). Testing this molecule to inhibit the activity of the SpnHl was done for the first time and it gave good results. It is noted from the chemical formula of the molecules used in the inhibition that the molecules of aromatic nature with few rings that possess hydrogen bonds have a high affinity for bonding with the enzyme by replace these molecules and affinity to form a hydrophobic interactions between enzyme and ligand.

It was noted that acetylsalicylic acid (aspirin®) gave weak inhibitory activity on enzyme although the result of the Schrödinger program was high. The inhibitory activity of this molecule which is classified as a non-steroidal anti-inflammatory drugs, varies in its effect between mammalian and bacteria, in mammals it has an effect as high activity of hyaluronidase and improves the motility of the sperms, but at the same time it reduces the amount of semen and the content of the sperms and increases the risk of hemorrhage by exceeding the dose recommended. While in bacteria, it has more affinity for binding with enzymes such as Hylase (Tanyıldızı & Bozkurt, 2003). As for the rest of the other molecules, their inhibitory activity of an enzyme varied as a result of their weak binding to the active site of the enzyme.

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