Isolation and purification of amylase from serum of patients with pancreatitis and comparing the biochemical properties with amylase purified from healthy people.

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Abstract

Back Ground: Amylases are a group of enzymes that hydrolyze starch into simple sugars. Amylase is secreted in the human body from saliva and the pancreas. Abnormal levels of the enzyme amylase indicate pancreatitis. Enzyme purification eliminates various proteins and other forms of biomolecules while restoring the majority of enzyme activity.

Objectives: Isolation and purification of the alpha-amylase enzyme from the serum of a patient with pancreatitis and a healthy human, and estimation of the values of Michalis constant Km as well as the maximum velocity Vmax to determine the affinity of the enzyme towards the substrate in both cases.

Materials and Methods: The enzyme was purified by several steps, including precipitation, by adding ammonium sulfate at a concentration of 30-70%, then dialysis. The extract was transferred through the separation column by gel chromatography containing Sephadex G100 gel.

Results: The gel separation chromatography results indicated the appearance of four protein bands, one of which (the third peak) belongs to the amylase enzyme. The specific activity of the enzyme in the last step after concentration of the product was 38 units /gm for the patient and 11.62 U/gm for a healthy human. The yield was 44.67% and 50.53%, while the number of purification times was 9.11 and 9.3 for the patient and the healthy human, respectively. The kinetic constants (Km and Vmax) were estimated using the Leinweaver-Burke plot, Vmax for the patient and the healthy human was 149.3 and 83.3, respectively. The Km for the patient and the healthy human was 1.39 and 2.56, respectively.

Conclusion: It is inferred from the results that the affinity of the enzyme to bind to the substrate of patient with pancreatitis is higher than that of the healthy human.

Keywords: Pancreatitis, alpha-amylase, Serum, Extraction, Purification, kinetic constants.

Introduction

Pancreas have two main functions are the production of hormones and the production of a number of enzymes, these enzymes It is excreted from the pancreas into the blood through a small tube is the pancreatic duct (1). Pancreatic enzymes (trypsin, chymotrypsin, lipase, lactase, amylase (figure 1) and sucrase) are chemicals made by the cells of the pancreas. The pancreas normally secretes 8 cups of pancreatic juice into the duodenum each day, which contains pancreatic enzymes that help digestion. A total 90% of pancreatic cells are dedicated to the production of pancreatic enzymes, and these cells are known as enzyme-producing cells (acinar cells). Pancreatitis is defined as a sudden inflammation of the pancreatic tissue, usually accompanied by severe abdominal pain and elevated serum concentrations of pancreatic enzymes, and the cause in most cases is gallstones or alcoholic stones. (2)

Amylase (EC 3.2.1.1) is one of the digestive enzymes that help the body digest and break down carbohydrates. The role of amylase is to stimulate the breakdown of carbohydrate compounds, by converting starches into smaller carbohydrate molecules such as maltose. Diarrhea result from a lack of amylase, also diarrhea result from the effects of undigested starch in the colon. Many medical conditions affect amylase levels in the blood, as abnormal levels of the enzyme amylase may indicate pancreatitis or other medical problems related to it, (3). Amylase is an enzyme that rises in the blood during pancreatitis (4). There might be an increase in the serum amylase activities in other intra-abdominal inflammatory conditions as well as salivary gland diseases when is impaired renal clearance because of renal insufficiency or serum amylase (in which amylase binds to immunoglobulins or polysaccharides to create large molecular weight complexes) (5)

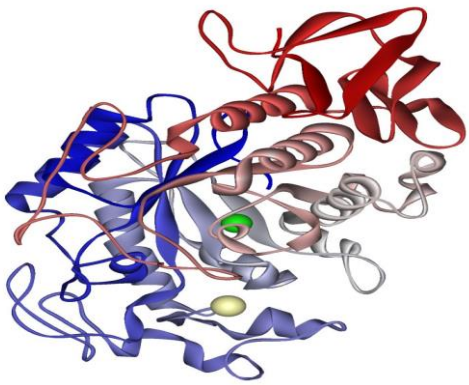

Figure (1): 3D image of Alpha-amylase (6)

Amylase is a hydrolysis enzyme that breaks the glycosidic bond at the alpha 1-4 sites in starch and glycogen (figure 2). The optimum pH of the human amylase enzyme is (6.7-7) within a temperature of 37°C or 40°C. The activity of this amylase continues until 50°C. Beta-amylase, alpha-amylase, and gamma-amylase are the 3 types of amylases. This enzyme is found in the salivary glands of humans. and the pancreas as well as some mammals (7). The pancreas and salivary glands cause the amylase enzyme to break down food starch into double and triple sugars that are converted by other enzymes into glucose to provide the body with energy (8). Alpha-amylase enzyme does not complete its work in the absence of calcium, its optimum pH 6.7-7 (8) While beta-amylase, which is found in some types of bacteria and fungi, the optimum pH for it is between 4-5 (9,10). Gamma-amylase has an optimum PH of about 3 (11).

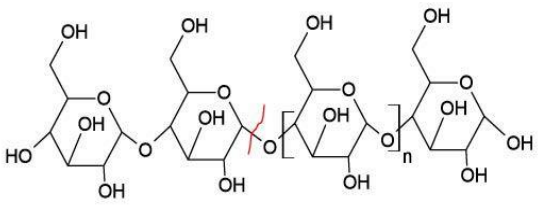

Figure (2): alpha amylase (the glyosidic bonds are alpha 1-4)

Over the years, various enzyme purification approaches were created. The physicochemical features regarding the enzyme of interest are used in conventional purification techniques. These techniques have been created in the 20th century to elucidate enzyme processes and solve protein 3D structures, yet they also appeared to be useful in the manufacture of highly pure biocatalysts. (12,13) Throughout enzyme purification, a purification approach could be used to describe the enhancement in specific activity and yield of the enzyme following each purification stage. In addition, the purification factor (the specific activity achieved following a purification stage divided by the starting materials) gives information about each step's "efficiency." In the case when a pure enzyme is acquired, the amount of that enzyme contained in the starting material is also determined (14). Purification leads in the recovery of the majority of enzyme activity (high yield) as well as the elimination of various "contaminating" proteins and other forms of (bio) molecules (a strong increase in specific activity). Ammonium sulfate fractionation is a common first step in conventional enzyme purification techniques. Individual proteins precipitate at varying concentrations of ammonium sulfate, which allows for this form of fractionation (14). One of the most extensively utilized approaches for enzyme purification is IEC. It categorizes protein molecules based on their charge differences (15). Sample molecules do not adhere to the column in gel filtration, also known as molecular sieve or size exclusion chromatography (SEC), yet are separated depending on their relative shape and size (16). One of the most effective methods for purifying proteins is bio-affinity chromatography. Because it makes use of specific, reversible interactions between biomolecules, the approach has a high selectivity (17).

Materials and methods

All procedures have been carried out at Biochemistry lab in College of Science - University of Fallujah. Potassium dihydrogen phosphate, Dipotassium hydrogen phosphate and Ammonium sulfate were purchased from BDH. Sephadex G100 was purchased from Sigma-Aldrich. Total protein kit from Biolabo and Alpha-Amylase kit from Taytec.

Sample collection

A sample of a person suffering from pancreatitis with no other symptoms was obtained from the laboratories of Fallujah Teaching Hospital. In addition, a healthy individual was included as a control. Serums were separated and kept at -20 °C until use.

Total Protein estimation

The protein content was determined using the biuret method biolabo kit.

Amylase Assay

The activity of alpha amylase was determined using the colorimetric method taytec kit.

Determination of Specific Activity

Specific activity of the amylase was specified with the use of the formula below.

Specific activity = Enzyme activity (units/ml) / Protein concentration (mg/ml)

Precipitation using ammonium sulfate

Solid ammonium sulfate has been added gradually to serum with constant stirring until 30-70% (18) percent saturation was obtained. The suspension was held at 4° for 6 hr. the precipitate collected by centrifuging at 8000xg for 30 min at 0°. The supernatant was discarded. The precipitate was dissolved in a 5ml of cold 20 mM phosphate buffer, pH 6.8.

Membrane screening (dialysis)

The process of membrane sorting of the solution resulting from the previous step was carried out to get rid of ammonium sulfate using a dialysis bag (a semi-osmotic membrane) in a high-concentration sugar solution and left in the fridge for 24 hours at a temperature of 4°C. The enzyme activity and protein concentration have been evaluated. (19)

Preparation of sephadex G-100 gel:

The gel was prepared according to the supplied company's instructions, with the use of potassium phosphate buffer (20 mM, pH=6.8), after that, the gel was suspended in the same buffer, degassed with a vacuum pump, and packaged gently in a glass column with dimensions of (2.5×90) cm. The column was equilibrated with the use of the same buffer that was utilized in gel suspension.

Enzyme separation through sephadexG-100 column:

The enzyme solution from the precipitation step was gently applied to the gel surface after concentration, and the elution was accomplished with the use of potassium phosphate buffer (20 mM, pH 6.8) at a flow rate of 20 ml/hour 3 ml for each one of the fractions (20). The enzymatic activity of fractions was detected at 280nm, after that the activation parts were collected and the activity, volume, and protein content were quantified, then separated into vials and stored at a temperature of -20°C for the subsequent tests.

Determination of Km and Vmax values:

Different concentrations of substrate (0.265-2.65 mM) were prepared to measure the value of Michalis - menten constant (Km) and maximum velocity (Vmax). The values of Michalis - menten constant (Km) and maximum velocity (Vmax) were measured by Lineweaver- Burk reciprocal plot, the relation between (1/Vo) and (1/S) were drawn. The point of intersection of the y-axis (1/Vmax) and the point of intersection of the x-axis (1/(S)) are defined to find the Vmax and Km, respectively

Results

Purification of the amylase enzyme

The first step in purification was to concentrate the enzyme with ammonium sulfate salt with a graduated saturation of 30-70%, (21) the precipitate was collected from a centrifuge, then dissolved in phosphate buffered solution (pH 6.8) and dialysis with a concentrated solution of sugar. It was assessed the enzyme activity, size, and protein content. Enzyme specific activity increased to 18.58 U/mg after dissolving precipitate and dialyzed supernatant, with a purification fold of 4.46 and a yield of 59.49%, compared to crude extraction for patient (table 1), whereas enzyme specific activity was 6.85 U/mg, with a purification fold of 5.49, and a yield of 65.01% for healthy human (table 2). In the second step of purification, the enzyme solution produced from ion precipitation step was passed through gel filtration using sephadex G-100 column (90×2.5) cm that equilibrated with phosphate buffer 20mM (pH=6.8), the fractions (each part 3ml) were collected from column at flow rate of 2ml / 5 minutes and measured at 280nm absorbency as well as an estimate Enzyme activity for the parts that give protein results. Four peaks of protein with one peak of enzymatic activity was appeared (figure 3). The parts with enzymatic activity were collected and concentrated by dialysis. The specific activity was 38.0 U/mg, with a purification fold 9.11 and enzymatic yield 44.67% for patient (table 1), and the specific activity was 11.62 U/mg, with a purification fold 9.3 and enzymatic yield 50.53% for healthy human (table 2).

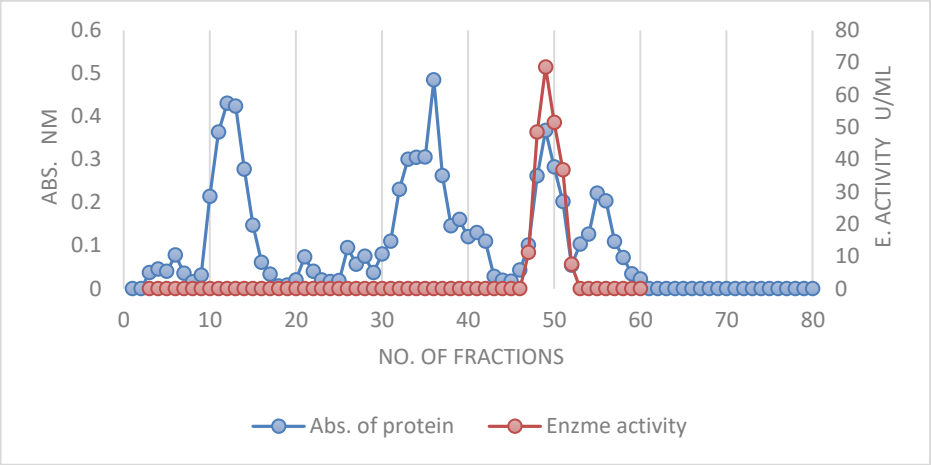

Figure (3): Gel filtration chromatography using sephadex G-100 column with dimensions (90×2.5) cm for purification the amylase from serum of a patient with pancreatitis equilibrated with phosphate buffer 20mM (pH=6.8), flow rate 24ml/ hr. and fraction volume 3ml.

Table (1): The purification steps of amylase enzyme from the serum of a patient with pancreatitis

Step	Volume ml	Enzyme Activity U/L	Total protein gm/L	Specific activity U/gm	Total activity U	Purification	Yield %
Serum	2	357.3	85.7	4.17	714.6	1.00	100.00
Ammonium sulfate precipitation 70%	1.3	327	17.6	18.58	425.1	4.46	59.49
Sephadex G-100 collumn chromatography	2.4	133	3.5	38.00	319.2	9.11	44.67

Table (2): The purification steps of amylase enzyme from the serum of a healthy human

Step	Volume ml	Enzyme Activity U/L	Total protein gm/L	Specific activity U/gm	Total activity U	Purification	Yield %
Serum	2	81.2	65	1.25	162.4	1.00	100.00
Ammonium sulfate precipitation 70%	1.7	62.1	9.06	6.85	105.57	5.49	65.01
Sephadex G-100 collumn chromatography	2.2	37.3	3.21	11.62	82.06	9.3	50.53

Determination of Km and Vmax values of pancreatic amylase

the Michalis – Menten method shows The relationship between reaction velocity and substrate concentration (figures 4 a&b), the enzyme activity was measured using different concentrations of the substrate ranging from 0.265-2.65 mM, the results showed that the enzymatic activity increased until it became a constant value at a concentration approximately 2.12 mM due to the saturation of the active site of the enzyme with the substrate.

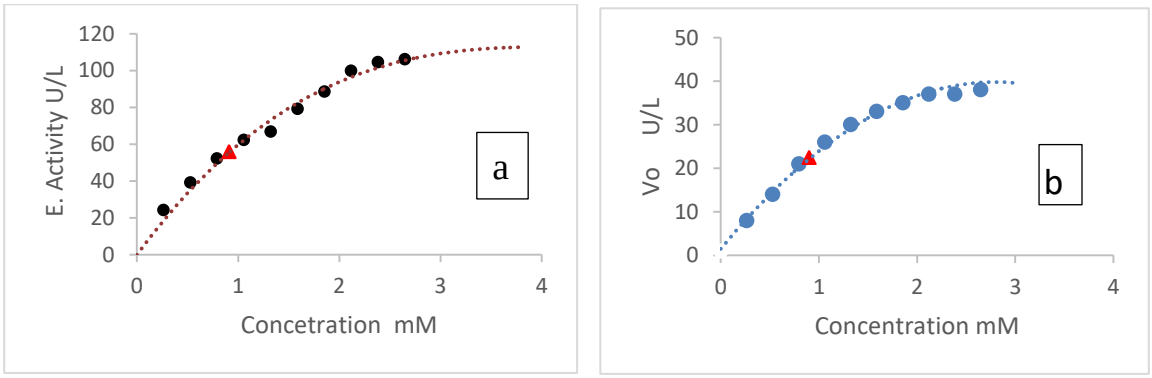

Figure (4): a- Relation between substrate concentration and activity of amylase enzyme in serum patients with pancreatitis. b- The relation between substrate concentration and activity of amylase enzyme in serum healthy human.

The values of Michalis – Menten constant (Km) and maximum velocity (Vmax) of pancreatic amylase purified from serum were estimated by using Lineweaver-Burk method, the Lineweaver-Burk diagram (figures 5 a&b) show the relationship between 1/Vo and 1/(S), the maximum speed value (Vmax) was 149.3U/L and Km was 1.39 mM for patient, while Vmax was 83.3 U/L and Km 2.56 mM for healthy human.

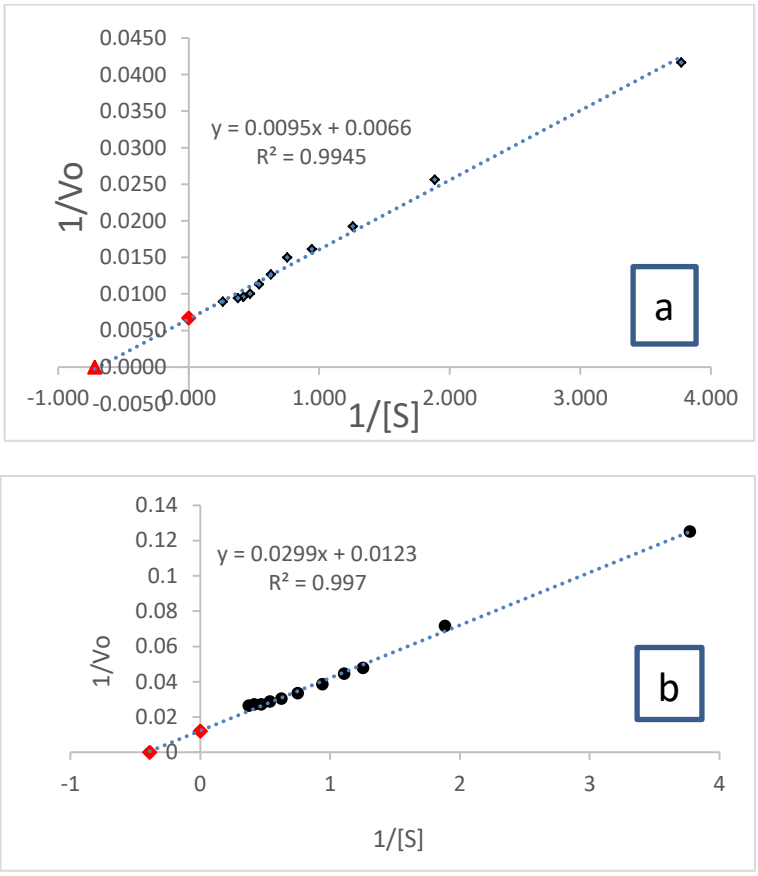

Figure (5): a- The Lineweaver-Burk plot for activity of amylase enzyme in serum pancreatitis patients. b- The Lineweaver-Burk plot for activity of amylase enzyme in serum healthy human.

Discussion

Two steps were done to purify the enzyme from serum including (precipitate of serum by ammonium sulfate and Gel filtration chromatography by using spadix G-100). Ammonium sulfate is the most used material in enzyme precipitation because it is cheap It does not affect the pH of the solution, has a high solubility in water, and does not affect the composition of the solution the enzyme (22). The presence of proteins and other compounds in the extract affects the optimum concentration of The sulfates needed for the precipitation process, which is the optimum concentration of ammonium sulfate used for precipitation an enzyme has to do with the number and distribution of charges and non-ionic and hydrophobic groups on the surface of a molecule Enzyme in addition to the size and shape of the enzyme, the sedimentation mechanism occurs by a phenomenon known as external salting out (by the tendency of the enzyme molecules to gather with each other due to the salt that works on Pulling the hydrated layer surrounding the enzyme molecule, which leads to the neutralization of the charges on the surface protein, lowering its solubility, and then precipitating it. (23). The membrane sorting process was applied for further purification in order to get rid of ammonium sulfate and impurities. The second step of purification is done by gel filtration chromatography (sephadex G100 column), gel filtration chromatography is a type of size exclusion chromatography that can be used to separate molecule in a sample into fractions with a specific size range, to remove all molecules larger than a specific size from a sample (24). The Km and Vmax results from the Lineweaver-Burk diagram suggest the pancreatic amylase enzyme in patients group tend to more than healthy group substrate.

Conclusions

A low Michaelis' constant indicates that the enzyme has a high affinity towards the substrate and the concentration of the enzyme (the lower the value of the constant, the more affinity the enzyme is with the reaction material). (25)

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عزل وتنقية انزيم الاميليز من مصل مرضئ التهاب البنكرياس ومقارنة الخصائص الكيميائية الحيوية مع الاميليز المنقى من الاشخاص الاصحاء

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الخلاصة

خلفية عن الموضوع : الاميليزات هي مجموعة من الانزيمات تحلل النشا الى سكريات بسيطة. يفرز الاميليز في جسم الانسان من اللعاب والبنكرياس. تشير المستويات غير الطبيعية من إنزيم الأميليز إلى التهاب البنكرياس. تؤدي تنقية الانزيم إلى التخلص من البروتينات المختلفة والأشكال الأخرى للجزيئات الحيوية مع استعادة غالبية نشاط الإنزيم .

الهدف من البحث: عزل وتنقيه انزيم الاميليز من مصل مريض مصاب بالتهاب البنكرياس وشخص سليم، وتقدير قيم ثابت ميكاليس Km والسرعة القصوى Vmax لتحديد مدى الفة الانزيم تجاه الركيزة في الحالتين.

المواد وطرق العمل: نقي الانزيم بعدة خطوات تضمنت الترسيب باضافة كبريتات الامونيوم بتركيز 30-70% ثم الفرز الغشائي (الديلزه) . نقل المستخلص خلال عمود الفصل بكروماتوغرافيا الهلام الحاوي على هلام سيفادكس G100.

النتائج: بينت نتائج كروموتوكرافيا الفصل بالهلام ظهور اربعة حزم بروتينية تعود احداها (القمة الثالثة) الى انزيم الاميليز، كانت الفعالية النوعية للانزيم في الخطوة الاخيرة بعد تركيز الناتج 38 وحدة /غم للشخص المصاب و 11.62 وحدة/غم للشخص السليم . كانت الحصيلة 44.67 % و 50.53% ، أما عدد مرات التنقية فكانت 9.11 و 9.3 للشخص المصاب والشخص السليم على التوالي. قدرت الثوابت الحركية (ثابت ميكليس والسرعة القصوى) باستخدام بياني لاينويفر-بيرك ، كانت قيمة السرعة القصوى Vmax للشخص المصاب والشخص السليم 149.3 و 83.3 على التوالي، وكانت قيمة ثابت ميكاليس Km للشخص المصاب والشخص السليم 1.39 و 2.56 على التوالي.

الاستنتاجات: يستدل من النتائج اعلاه ميل الانزيم للارتباط بالمادة الاساس للشخص المصاب بالتهاب البنكرياس اعلى من الشخص السليم.

الكلمات المفتاحية: التهاب البنكرياس، انزيم الفا-اميليز، مصل الدم، استخلاص، تنقية، الثوابت الحركية.