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Pepsin, Papain and Hyaluronidase Enzyme Analysis: A Review

Trivedi Vishal*, Rathore R P S, Kamble P R, Goyal Manish, Singh N

Department of Quality Assurance, B.N. College of Pharmacy
Udaipur Rajasthan, India

ABSTRACT:

Enzymes are biocatalysts that increase the rate of otherwise slow reactions by decreasing the reactions activation energy, without undergoing any net change in their structures at the end of a reaction. They are mostly protein in nature. In this text enzyme analysis of pepsin and papain and hyaluronidase has been investigated to achieve the objectives of understanding the principle of enzyme assays and kinetics, action of an enzyme, effect of pH on enzyme activity effect of enzyme inhibitor on enzyme activity and enzyme importance in disease conditions.In this pepsin analysis in tissue preparation is studied as well as its action on suspension of egg. Various activity of papain such as proteolytic activity, endoesterolytic activity, amidolytic activity also studied. Enzyme-linked Immunosorbent Assay For Hyaluronidase (HAase) also been studied. Sandwich enzyme immunoassay is for the in vitro quantitative measurement of HAase in human serum, plasma, tissue homogenates and other biological fluids.

KEYWORDS: Pepsin Papain, Hyaluronidase Proteolytic, Enzyme analysis, Endoesterolytic, Amidolytic

***Corresponding author-:**
Vishal Trivedi
M.Pharma(2nd sem.) Department of Quality Assurance,
B.N. College of Pharmacy,
Udaipur (Rajasthan) India.
E Mail Id-vishaltrivediqa@gmail.com
Contact: 09829245123

TABLE OF CONTENT:

1. INTRODUCTION[1,2,3]

Enzymes are biocatalysts that increase the rate of otherwise slow reactions by decreasing the reactions activation energy, without undergoing any net change in their structures at the end of a reaction. They are mostly protein in nature and mediate all synthesis and degradation reactions carried out by living organisms. Ribozyme is a RNA molecule that has enzymatic properties. Enzymes catalyze almost all the biological reactions, converting a target molecule (the substrate) into product(s). Enzymes are considered as an integral part to biological systems because they allow the body to carry out reactions that normally would require too much energy to initiate.

An enzyme acts on a substrate to form an enzyme-substrate complex, which then forms a new product and the free enzyme. The active site of an enzyme, where the substrate binds, only recognizes the specific substrate and holds it in a set confirmation. The enzyme activity is a measure of the rate or velocity of the conversion of the substrate to the product per unit time by an enzyme. The enzymes generally work under mild conditions of temperature, pressure and pH that decrease the energy requirement.

The following terms are used to define enzyme more specifically:

- **Apoenzyme** - The protein portion of an enzyme is called the apoenzyme**.**
- **Cofactor -** It is the non-protein part of an enzyme. Cofactors can be loosely bound, **Coenzymes**, or tightly bound, **prosthetic groups**.
- **Holoenzyme**- The complete enzyme (apoprotein + cofactor) is termed the Holoenzyme.

Based on type of reaction:-

1. Oxidoreductase - catalyze a redox reaction
2. Transferase - transfer a functional group
3. Hydrolase - cause hydrolysis reactions
4. Lyase - break C-O, C-C or C-N bonds
5. Isomerases - rearrange functional groups
6. Ligase - join two molecules

Formation of the enzyme-substrate complex:-

First step in an enzyme catalyzed reaction

E + S ES

Enzyme Substrate Complex

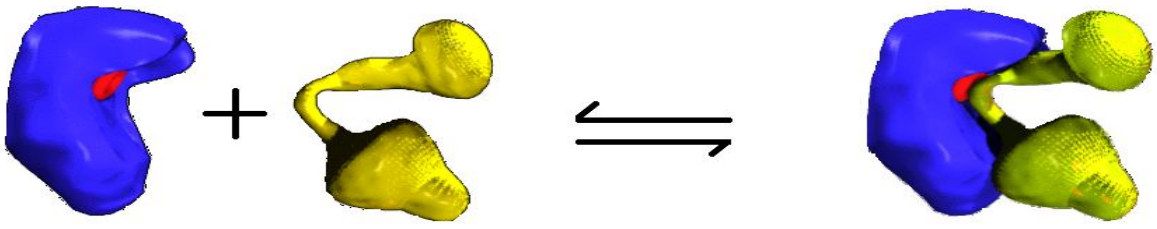


Figure no. 1-Enzyme Substrate Complex

2. PEPSIN ANALYSIS:[3,4]

2.1 INTRODUCTION

Pepsin is a proteolytic enzyme from acid protease family.

2.1.1 What is Pepsin?

Pepsin is a enzyme that aids in digestion. It was discovered in 1836. Pepsin is found in the stomach and is the main digestive aid, breaking down food proteins into peptides. Pepsin is an enzyme, protein and catalyst, which means that it accelerates chemical reactions.

2.1.2 How Does It Work?

It splits protein into smaller pieces called peptides. These are easier for the stomach to work with it.

2.1.3 Does Pepsin Cause Ulcers?

Ulcers are caused by bacteria called Helicobacter pylori. The bacteria prevent protective mucus forming. This cause the pepsin to digest the stomach lining.

2.1.4 What Effect Do Medication Have on Pepcid?

Antacid can inhibit the stomachs ability to digest proteins. Aspirin, ibuprofen and naproxen sodium can cause the stomach to be susceptible to pepsin and acid.

2.1.5 Are there Medication That Contain Pepsin?

Yes, pepsin is one of the ingredients of Pepto- Bismol. Pepsi Cola was named after pepsin, which was one of the ingredients.

2.1.6 Pepsin (Proteolytic Enzyme)

Synonyms	-	Pepsin A : Pepsin NF: pepsinum : Puerzym
Mol.Weight	-	34500
CAS No.	-	9001-75-6
Appearance	-	White to cream coloured, fine amorphous powder, with faint Characteristic odour (meaty), not offensive smell
Chemical Properties	-	Slightly soluble in water , insoluble in ethanol an ether.
Application	-	Proteolytic enzyme, digesting.

Mechanism - Used I combination with cathepsin and protein bound HCL and strongly increases the production of gastric juice.

Specification

- Sample Standard of 1:15000 NF
- Pepsin– 15000 NF μ/g min.
- Moisture – 5% max.
- E.coil – Negative
- Salmonellae – Negative
- Mould and Yeast – 100/g max

Packing : in firbe drum of 25 kgs net each, with separate inner PE Liner

Storage: Condition to Avoid keep container tightly closed. Store in cool, dry, well- ventilated area. Do not expose to heat. Refrigeration is recommended for extended storage periods.

Incompatibilities: Tannin, alkalis, salts of heavy metals, strong oxidizers. Moisture, heat, flames ignition sources and incompatibles.

Quantity available: 10,000 -15,000 kgs mounthly.

Remarks: The product can be supplied as per customer's requirement.

2.2 ANALYSIS

Various methods for pepsin analysis is as follows:-

2.2.1 Experiment – A: - Analysis of pepsin in tissue suspension.[5]

Principle: - This enzyme, in an acid solution, breaks complex proteins down into peptones. Fibrin, was dyed in a 1% aqueous solution of amaranth red for 15 minutes. All surplus stain was then removed by thoroughly washing with water whose acidity was adjusted to pH = 3 with HCL. This gave a red fibrin whose color would not come out in an acid solution. Even the slightest digestion of the fibrin is then made apparent by a noticeable red color in the digested fluid.

Procedure:-

1. Adjust the tissue suspensions to an acidity whose pH = 2 with HCL.
2. 2 ml. tissue suspensions + colored fibrin +.5 ml. toluene.
3. Incubate 96 hours at room temperature.

Result: - If red colour is reaction mixer then pepsin is present in tissue suspension.

2.2.2 Experiment – B: - Determination of pepsin activity[6]

Materials

- Egg white (hard-boiled)
- Test tubes (6)
- Test tube rack
- Solutions:
    A. 1.0% Pepsin
    B. Pepsin (1.0%) in 0.4% hydrochloric acid
    C. 0.4% Hydrochloric acid
    D. Pepsin (1.0%) in 0.5% sodium bicarbonate
    E. 0.5% Sodium bicarbonate
    F. Distilled water
- Metric ruler
- Knife

Procedure: -

1. Label test tubes A to F.
2. Fill each tube 1/3 full (5 ml) with the corresponding solution.
3. Drop a small (2 mm) cube of egg white into each tube.
4. Incubate at room temperature for approximately 12 hours. (The speed of this reaction can be increased by using very thin strips of egg white and/or incubating at 30°C. Students might want to compare the action of papain or bromelin under similar conditions.)
5. Examine tubes for the presence of the egg white.

Results: -

After 12 hours, the egg white will be "digested" in tube B; while there appears to be little change in the other tubes. If allowed to incubate longer, the cube in tube D and possibly C will decrease in size. Thus pepsin activity is optimum at acidic pH

2.2.3 Experiment C [7,]

Principle - Pepsin enzymes are added to a milky colloidal suspension of egg albumen. As the pepsin enzymes digest the suspended particles of proteins, the mixture becomes more transparent. The absorbance changes in the reaction mixture are followed with a colorimeter. The pepsin content, for example in extracts of fruits, can be assayed by measuring the rate at which the solution of egg

albumen and extract becomes clearer.

Preparation of the egg albumen colloidal suspension (enzyme substrate)

- Separate the white of a single egg into a 250 ml beaker and add 150 ml tap water, stirring the mixture thoroughly. The mixture becomes quite cloudy as a result of the denaturation of some of the egg albumen by the water.
- Place the beaker on a tripod and gauze and heat with a bunsen until the mixture boils and stirring it regularly.
- Allow the mixture to cool, then decant it through two or three layers of muslin into another beaker. This creates a homogenous milky colloidal solution.

Procedure: -

1. Select a test-tube that fits into the colorimeter. Add 2 ml of an appropriate buffer (e.g. pH 7), followed by 2 ml of the albumen substrate solution and 1 ml of test sample of pepsin.
2. Mix the contents of the colorimeter tube, and place it in the colorimeter (previously zeroed using a tube with 4 ml buffer and 1 ml of fruit extract). Read the absorbance and note the time.
3. Place the reaction mixture(s) in a water bath at 30°C to promote the activity of any protease enzymes present.
4. Take further absorbance readings at regular time intervals (say every 5 or 10 minutes), until no further change (decrease) in absorbance is detected.
5. Plot a graph of the change (decline) in absorbance against time. Measuring the time taken for a 50% reduction in absorbance value gives an indication of the protease activity of the original fruit extract.

3. PAPAIN ANALYSIS

3.1 INTRODUCTION[8,9,10]

Papain is a proteolytic enzyme from Cysteine protease family. Responsible for breaking down proteins, papain is an enzyme present in papaya and is used for breaking down meat fibres. Papain is another type of proteolytic enzyme. Unlike, pepsin and trypsin, which are synthesized in the human body, papain is typically found in plants. Papain obtain from fruits including papaya and pineapple, Papain also has a number of food processing applications. It is as use papain as a meat tenderizer, since the enzyme can break down muscle fibers in tough meat, leaving it tender. It is also used to extract fish oil

rich in vitamins A and D, to make into supplements.

Definition: Papain is a proteolytic enzyme preparation derived from fruits of papaya. It may contain lactose or dextrin.

Enzyme activity: the enzyme activity of papain in not less than 300,000 , units per gram.

Description: Papain occurs as whit to light yellow-brown powders. It is odourless or has slight characteristic odors

Identification:

1. To 10ml of a liquid including 20% of powdered skim milk, adjusted to pH 5.5 with diluted acetic acid (3/50), add0.01g of papain and warm to 37. The liquid cogulates.
2. The solution of papain(1/500)exhibits absorption maximum at a wavelength of 270to280nm.

Purity-

(1) **Heavy metals-** Not more than 40µg/g as pb(0.50g, method 2, control solution Lead Standard Solution 2.0ml)

(2) **Lead-** Not more than 40µg/g as Pb(1.0g, Methed 1)

(3) **Arsenic-** Not more than 4.0µg/g as As_2O_3 (0.50g, Method3, Apparatus B

Microbial Limits – proceed as directed under the microbial

Limit test- the total viable aerobic count is not more than 50,000/g and no E.coli is observed

3.2 ANALYSIS:

Various Experiments for papain analysis are as follows:

3.2.1 Experiment – A: - In this various activity of papain is determine [11]

Proteolytic activity assays

1. Proteolytic assays were made using casein as substrate.
2. The reaction mixture was prepared by mixing 0.1 ml of the papain test sample with 1.1 ml of 1% casein containing 12 mM cysteine in a 0.1 M Tris–HCl buffer (pH 8.5).
3. The reaction was carried out at 420C and stopped 2 min later by the addition of 1.8 ml of 5% trichloroacetic acid (TCA).
4. Each test tube was centrifuged at 3000 g for 20 min and the absorbance of the supernatant measured at 280 nm.
5. An arbitrary enzyme unit (Ucas) was defined as the amount of enzyme (g) that produces an

increase of one absorbance unit per minute in the assay conditions.

Measurement of endoesterolytic activity: -

These assays were carried out by the Silverstein's Experiment modified to reach optimal conditions of the enzyme. The activity was studied using N-α-Cbz-L-Gln-p-nitrophenyl esters of some L-amino acids (Ala, Asn, Gln, Gly, Ile, Leu, Trp, Pro, and Val) as substrates. Assays were made at 370C in 0.1 M Tris–HCl buffer (pH 8.0) containing 2 mM EDTA, 25 mM cysteine and 1 mM of each substrate in the reaction mixture. Liberation of p-nitrophenol was followed spectrophotometrically at 405 nm in an UV-visible spectroscopy system equipped with a chamber termostatized at 370C.

An arbitrary enzyme activity unit (Ucbz) was defined as the amount of protease (g) that released one micromol of p-nitrophenolate per min in the assay conditions. To determine the micromoles of p-nitrophenolate produced during the reaction, a standard curve with p-nitrophenol (5–50 mM) in 0.1 M Tris–HCl buffer pH 8.0 containing 5% acetonitrile was carried out.

Measurement of amidolytic activity: -

Amidolytic activity was determined by hydrolysis of L-pyroglutamil-L-phenylalanyl-L-leucine-p-nitroanilide (PFLNA). This assay was performed using a solution of 1 mM PFLNA in dimethyl sulfoxide (DMSO). The reaction mixture contained

- 1.5 ml of 0.1 M phosphate buffer, pH 6.5,
- 0.3 M KCl,
- 10-4 M EDTA,
- 0.003 M dithiothreitol (DTT),
- 0.18 ml substrate,
- and 0.12 ml papain test sample

The p-nitroaniline released at 370C was detected spectrophotometrically at 410 nm. An arbitrary enzyme activity unit (UPFLNA) was defined as the amount of protease (g) that released one micromol of p- nitroaniline per min in the assay conditions.

3.2.2 Experiment – B: - Papain Proteolytic Activity by Spectrophotometric method [12]

Reagents: -

(a) Sodium phosphate solution.—0.05M. Dissolve 7.1 g an hydrous Na2HPO2 in enough H2O to make 1 L. Add drop of toluene as preservative.

(b) Citric acid solution.—0.05M. Dissolve 10.5 g citric Acid.H2O in enough H2O to make 1 L. Add drop of toluene as preservative.

(c) Casein substrate.—Disperse 5g Hammersten-type casein in 250 mL 0.05M Na2HPO4. Place in boiling water bath 30 min with occasional stirring. Cool to room temperature and add 0.05M citric acid to pH 6.0 ± 0.1. Stir solution rapidly and continuously during addition of citric acid to prevent precipitation of casein. Dilute to 500 mL with H2O. Prepare fresh daily.

(d) Phosphate-cysteine disodium ethylenedinitrilotetraacetate buffer solution.—Dissolve 3.55 g Na2HPO4 in 400 mL H2O in 500 mL volumetric flask. Add 7.0 g Na2H2EDTA and 3.05 g cysteine×HCl×H2O. Adjust to pH 6.0 ± 0.1 with 1M HCl or 1M NaOH and dilute to volume with H2O. Prepare fresh daily.

(e) Trichloroacetic acid (TCA) solution.— 30%. Dissolve 60 g trichloroacetic acid in H2O and dilute to 200 mL with H2O.

(f) Papain standard solution.—Accurately weigh 100 mg USP Papain Reference Standard in 100 mL volumetric flask and add buffer solution, (d), to dissolve. Dilute to volume with buffer solution, (d). Further dilute 4 mL of this solution to 100 mL with buffer solution. Use within 30 min of preparation.

Preparation of Test Solution

Accurately weigh test portion containing activity equivalent to 100 mg reference standard and proceed exactly as in preparation of papain standard solution.

Determination

1. Into each of 12 glass-stoppered 100 mL volumetric flasks, pipet 25 mL casein substrate.
2. Label flasks in duplicate (tests are run in duplicate except for blank) S1, S2, and S3 for papain standard solution and U2 for test solution. Label remaining 4 flasks (blanks) S1B, S2B, S3B, and U2B.
3. Add 5, 2.5, and 0 mL buffer solution, respectively, to flasks S1, S2, and S3 and also to their respective blanks S1B, S2B, and S3B. To U2 and U2B add 2.5 mL buffer solution.
4. Place all flasks in 40°C water bath and al low 10 min to reach bath temperature.
5. Into each of duplicate flasks S1, pipet 5 mL papain standard solution, noting 0 time upon release of pipet with simultaneous swirling of flask to mix. Stopper and replace in bath.
6. Into 2 flasks labeled S2, pipet 7.5 mL papain standard solution and proceed as before.
7. Repeat for 2 flasks S3 to which 10 mL papain standard solution is added and for 2 flasks U2 to which 7.5 mL test solution is added.
8. After exactly 60 min add 15 mL 30% TCA to all 12 flasks and shake vigorously. With 4 flasks to which no standard or unknown solutions were added, prepare blanks by pipetting, respectively, 5 mL (S_{1B}), 7.5 mL (S_{2B}), and 10 mL (S_{3B}) papain standard solution, and 7.5 mL

(U_{2B}) test solution.

9. Replace all flasks in 40°C bath 30–40 min and let precipitated protein fully coagulate.
10. Filter through Whatman No. 42, or equivalent, paper, refiltering ca first half of filtrate through same filter (filtrates must be completely clear).
11. Read Abs of filtrates at 280 nm against respective blanks.
12. Plot readings for S_1, S_2, and S_3 against enzyme concentration of each corresponding level in terms of mg/mL of 50 mL total test mixture.
13. By interpolation from this curve, taking into consideration dilution factors, calculate potency of test portion in USP Units of Papain by the following formula-

Activity/mg = C x (100/W) x (100/4) x (50/7.5) x U,

Where C = mg/mL obtained from standard curve,

W = mg test portion, and

U = activity of reference standard in units/mg.

Definition of Unit

One unit of papain activity represents activity which releases equivalent of 1 mg tyrosine from specified casein substrate, under conditions of assay and at enzyme concentration which liberates 40 mg tyrosine/mL test solution. USP Papain contains =6000 papain units/mg.

3.2.3 Experiment – C[13,14,15]

Materials

- Gelatin (Knox, Jello)
- Beaker (150 ml)
- Balance or teaspoon
- Stirring rods (3)
- Test tubes (2)
- Test tube rack
- Beaker of ice water
- Hot plate
- Distilled water (100 ml)

Procedure

1. Prepare a gelatin solution by heating 1 teaspoon (3.0 g) of gelatin in 100 ml distilled water until

dissolved. (Gently mix, do not boil.) Cool to room temperature.

2. Pour meat tenderizer into one of the two test tubes until it fills approximately 0.5 cm of the tube. Label this tube as P. Do not put meat tenderizer in the other tube.
3. Fill each test tube 1/3 full (5 ml) with the gelatin solution. Mix gently.
4. Place tubes in ice water for 10 minutes.
5. Remove from ice bath and note the degree of gelatinization.

Results

The tube without meat tenderizer (papain) will contain firm gelatin. Tube P which contains papain will be almost liquid.

4. HYALURONIDASE ANALYSIS

4.1 INTRODUCTION[16]

The enzyme is widely distributed in animal tissues but is found in great concentration in the bovine and ovine testes. It is also produced by a number of bacteria.

- **Formula** : $C_{2455}H_{3775}N_{617}O_{704}S_{21}$
- **Mol. Mass** : 533870.9g/mol
- **Activity** : More than 300U/mg
- **Appearance** : White Freeze dried powder.
- **Solubility** : Distilled Water.

By catalyzing the hydrolysis of hyaluronan, a constituent of the interstitial barrier, hyaluronidase lowers the viscosity of hyaluronan, thereby increasing tissue permeability. It is, therefore used in maedicine in conjunction with other drugs to speed their description and delivery. Commom applications are also increases the absorption rate of parentral fluids given by hypodermoclysis, and is an adjunct in subcutaneous urography for improving resorption of radiopaque agents. Hyalurobnidase is also used for extravasation of hyperosmolar solutions.

Purity:

Lead not more than 5mg/kg

Determine using atomic absorption technique appropriate to the specified level. The selection of the sample size and the method of sample preparation may be based on the principles described in volume 4.

Microbiological Criteria:

- **Salmonell spp.** : Absent in 25g sample.

- **Total colifroms** : Not more than 30/g
- **E.coli** : Absent in 25g sample

Determine using procedures described in volume 4.

Antibiotic activity:

Absent in preparations from microbial sources.

4.2 ENZYME ANALYSIS:

4.2.1 Experiment–A:-Enzyme-linked Immunosorbent Assay For Hyaluronidase (HAase) [17]

Scope

Sandwich enzyme immunoassay is for the in vitro quantitative measurement of HAase in human serum, plasma, tissue homogenates and other biological fluids.

Materials

1. Pre-coated, ready to use 96-well strip plate -1
2. Standard (freeze dried) -2
3. Detection Reagent A (green) -120µL
4. Detection Reagent B (red)- 120µL
5. TMB Substrate- 9mL
6. Wash Buffer (30 × concentrate)- 20mL
7. Plate sealer for 96 wells -4
8. Standard Diluent -20Ml
9. Assay Diluent A (2 × concentrate)- 6mL
10. Assay Diluent B (2 × concentrate) -6mL
11. Stop Solution- 6mL
12. Microplate reader with 450 ± 10nm filter.
13. Precision single or multi-channel pipettes and pipette tips with disposable tips.
14. Eppendorf Tubes for diluting samples.
15. Deionized or distilled water.
16. Absorbent paper for blotting the microtiter plate.
17. Container for Wash Solution
18. The Standard, Detection Reagent A, Detection
19. Reagent B and the 96-well strip plate should be stored at -20oC

Test principle

The microtiter plate has been pre-coated with a monoclonal antibody specific to HAase. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for HAase. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. Only those wells that contain HAase, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color.

The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of HAase in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Sample collection and storage

For Serum Use a serum separator tube and allow samples to clot for two hours at room temperature or - overnight at 4oC before centrifugation for 20 minutes at approximately 1000×g. Assay freshly prepared serum immediately or store samples in aliquot at -20oC or -80oC for later use. Avoid repeated freeze/thaw cycles.

For Plasma Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes- 1000×g at 2 - 8oC within 30 minutes of collection. Remove plasma and assay immediately or store samples in aliquot at -20oC or -80oC for later use. Avoid repeated freeze/thaw cycles.

For Tissue homogenates - The preparation of tissue homogenates will vary depending upon tissue type. For this assay, tissues were rinsed in ice-cold PBS(0.02mol/L, pH 7.0-7.2) to remove excess blood thoroughly and weighed before homogenization. Minced the tissues to small pieces and homogenized them in 5-10 mL of PBS with a glass homogenizer on ice(Micro Tissue Grinders woks, too). The resulting suspension was sonicated with an ultrasonic cell disrupter or subjected to two freeze-thaw cycles to further break the cell membranes. After that, the homogenates were centrifugated for 5 minutes at 5000×g. remove the supernate and assay immediately or aliquot and store at = -20oC.

For Other biological fluids - Centrifuge samples for 20 minutes at 1000×g. Remove particulates and assay immediately or store samples in aliquot at -20oC or -80oC for later use. Avoid repeated freeze/thaw cycles.

Reagent preparation

Standard - Reconstitute the Standard with 1.0 mL of Standard Diluent, kept for 10 minutes at room

temperature, shake gently(not to foam). The concentration of the standard in the stock solution is 5,000pg/mL. Please prepare 7 tubes containing 0.5mL Standard Diluent and produce a double dilution series according to the picture shown below. Mix each tube thoroughly before the next transfer. Set up 7 points of diluted standard such as 5,000pg/mL, 2,500pg/mL, 1,250pg/mL, 625pg/mL, 312pg/mL, 156pg/mL, 78pg/mL, and the last EP (Eppendorf) tubes with Standard Diluent is the blank as 0 pg/mL.

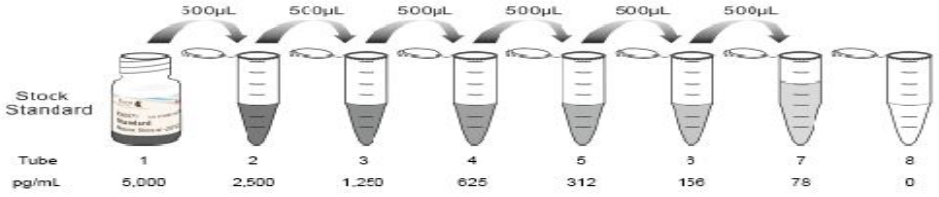


Figure 2-: Dilution of standard

Assay Diluent A and Assay Diluent B - Dilute 6mL of Assay Diluent A or B Concentrate(2×) with 6mL of deionized or distilled water to prepare 12 mL of Assay Diluent A or B. The prepared working dilution can't be frozen.

Detection Reagent A and Detection Reagent B - Briefly spin or centrifuge the stock Detection A and Detection B before use. Dilute to the working concentration with working Assay Diluent A or B, respectively (1:100).

Wash Solution - Dilute 20mL of Wash Solution concentrate (30×) with 580mL of deionized or distilled water to prepare 600 mL of Wash Solution (1×).

TMB substrate - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Assay procedure

1. Determine wells for diluted standard, blank and sample. Prepare 7 wells for standard, 1 well for blank. Add 100µL each of dilutions of standard, blank and samples into the appropriate wells. Cover with the Plate sealer. Incubate for 2 hours at $37^{O}C$
2. Remove the liquid of each well, don't wash.
3. Add 100µL of Detection Reagent A working solution to each well. Incubate for 1 hour at $37^{O}C$ after covering it with the Plate sealer.
4. Aspirate the solution and wash with 350µL of 1× Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let it sit for 1~2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent

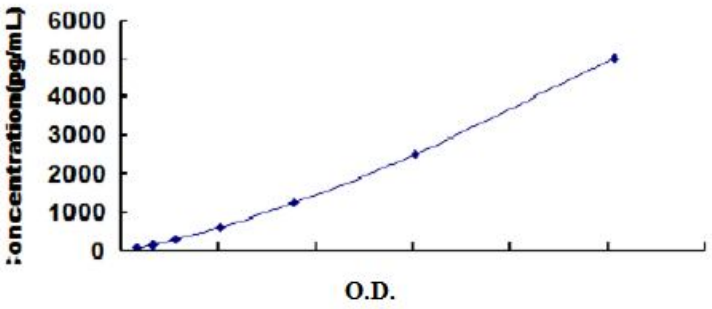
paper. Repeat 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

5. Add 100µL of Detection Reagent B working solution to each well. Incubate for 30 minutes at 37^{O}C after covering it with the Plate sealer.
6. Repeat the aspiration/wash process for five times as conducted in step 4.
7. Add 90µL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for 15 - 25 minutes at 37^{O}C (Don't exceed 30 minutes). Protect from light. The liquid will turn blue by the addition of Substrate Solution.
8. Add 50µL of Stop Solution to each well. The liquid will turn yellow by the addition of Stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450nm immediately. A microplate plate reader with a bandwidth of 10nm or less and an optical density range of 0-3 O.D. or greater at 450 ± 10nm wavelength is acceptable for use in absorbance measurement.

Calculation of results

Average the duplicate readings for each standard, control, and samples and subtract the average zero standard optical density. Create a standard curve on log-log graph paper, with HAase concentration on the y-axis and absorbance on the x-axis. Draw the best fit straight line through the standard points and it can be determined by regression analysis. Using some plot software, such as curve expert 1.30, is also recommended. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

In order to make the calculation easier, we plot the O.D. value of the standard (X-axis) against the known concentration of the standard (Y-axis),



Assay procedure summary

1. Prepare all reagents, samples and standards;
2. Add 100µL standard or sample to each well. Incubate 2 hours at 37°C;
3. Add 100µL prepared Detection Reagent A. Incubate 1 hour at 37°C;
4. Aspirate and wash 3 times;
5. Add 100µL prepared Detection Reagent B. Incubate 30 minutes at 37°C;
6. Aspirate and wash 5 times;
7. Add 90µL Substrate Solution. Incubate 15-25 minutes at 37°C;
8. Add 50µL Stop Solution. Read at 450nm immediately

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