University of Jordan
Faculty of Medicine

Medical Committee
The University of Jordan

Introduction to

BIOCHEMISTRY

Lecture #: (.....14.....)

■ Sheet ☐ Slides ☐ Other

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Price: .....

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Lecture Outline :

1- *Basic steps to prepare a protein for study by Extracting Pure Proteins from Cell*
2- *Salting In & Out*
3- *Dialysis*
4- *Column Chromatography*
5- *Size –exclusion chromatography*
6- *Molecular Sieve Chromatography*
7- *Affinity Chromatography*
8- *Affinity chromatography*
9- *Ion-Exchange Chromatography*
10- *Electrophoresis*
11- *Agarose VS Page*
12- *Isoelectric focusing*
13- *Protein Sequencing Edman Method*
14- *Protien sequencing Mass spectrometry*
15- *Protein Sequencing _ prediction from DNA &RNA*
16- *Imunoassays-Western Blot*
17- *imunoassays ELISA*

1- *Extracting Pure Proteins from Cell*

* Purification techniques focus (mainly) on size & charge and they could be purified on the basis of solubility and specific binding affinity.

-How to extract protein from cells? -The protein is found in a higher structure which is the cell, so we have to breakdown the cell by so the protein inside will be out (breaking down the higher structure to get smaller structures proteins part of it).

*Homogenization: Breakage down of the cell (membrane) By intensive blending of the cell found in certain liquid, buffering system which are not homogenous mutual related substances .so by cell breaking and stirring we will get the homogenous solution.

How homogenization occurs? Breaking down of the cells can be done by grinding e.g by : Potter–Elvejhem homogenizer, sonication(applying sound waves) , freezing and thawing technique Or detergents .These techniques result in crude protein not pure but it aids in reducing contaminations .

Potter–Elvejhem homogenizer: an instrument consist of test tube with a shaft inside used for breaking down cells (what dr said) . it is like the instrument we used in chemistry lab It consists of a cylindrical pestle (of glass, Perspex, or Teflon) that fits closely into a hard-glass test tube.

*Sonication : applying sound waves to some extent to the fluid the cells spreading inside result in breakage down of the cell because the sound waves in the fluid apply it is force in the overall area of the cell (through the fluid.

*Freezing And thawing: continuous rapid freezing and thawing can break the cell through which the water can be crystallized inside the cell membrane result in breaking down the cell.

*Detergents: It can make bonds with cell membrane constituents dissolving the cell membrane.

**Now after homogenization: the protein after the cells had been broken ,is found in a homogeneous surrounding Inside buffer, water…etc. so we got a mixture the protein is inside by a homogenous way .Now , How to get the protein of interest outside of the mixture ?? it is done initially by centrifugation .

*deferential Centrifugation done by moving a big wheel in a specific speed in a rotational movement higher than the gravity speed , big molecules will be precipitated (at the beginning) , by increasing the speed smaller one will be participated .(Deferential) separate certain organelles refer to slides but dr didn't say anything about bit they are in the slides ?? . (600 *g<=600 times gravity:* unbroken cells & nuclei; 15,000 here is smaller so it recommends higher speed to be participated *g:* mitochondria; 100,000 *g:* ribosomes and membrane fragments)

- Now we got a mix of proteins together with other contaminants so we have to start in purification.

*Here there are some methods used to purify proteins some of them gives a pure protein, some gives crude proteins. Rottenly, we start with purification techniques which give the crude mixture cause we

can’t choose something specific while we got for example a high amount of proteins .. So we start gradually from lower to higher techniques to decrease the level of contamination (unless we got a small mixture of proteins) .

** Salting in And Salting out:

Flashback > are proteins soluble in water? Yes. Remember: Hydrophilic AAs was accumulating at the exterior of the protein especially charged making interaction with water (bonding). while hydrophobic stack inside.

**summary: Usually the protein of interest is in high concentration in the solution so; it already did make lot of bonds with water. Contaminants are in lower concentration with less frequent binding pattern with water. Once we start adding salt to the water. Salt easily get ionized in water. The medium got ionized. (Enhancing the polarity of the medium) ➔ stabilizing the charge on the proteins as it is ➔ the solubility of proteins increase in water till a certain limit. Now, is Salting Out, Once the ions of salt become more than desirable (concentration of Salt higher than proteins) it‘ll bind water effectively more than protein. So the amount of water available for the protein is decreased since it is bonded to salt ➔ the proteins start to precipitate ➔ once they are precipitated they start to form bonds between each other. Hydrophobic AAs makes hydrophobic interaction with other in precipitated proteins. The proteins of lower solubility in water (the number of bonds b/w it and water) as protein with 3 bonds once u breakdown one of them the solubility decrease by a high value not like that of 10 bonds in b/w ,1 bond removing aint make that effect .

Salting In

when salt is added to the protein in the buffer or water,salt easily gets ionized in water and so the medium gains ions among it and thus the polarity of the medium is enhanced,(+ve charge,and–ve charge are available so anything with a –/+ve charge will maintain that charge, so the charges on the protein will remain as is) and eventually this leads to an enhanced solubility of protein in water, slightly. However, that happens to a certain extent–limit-.(enhancing the solubility of the protein in water).

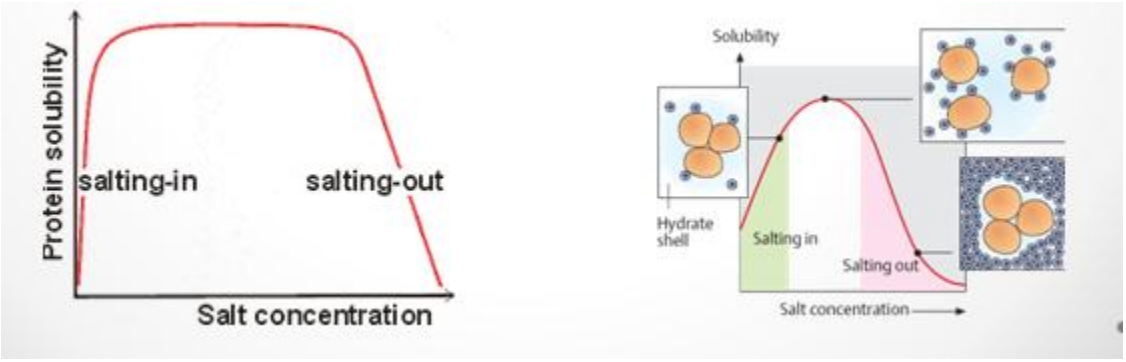
Again .Salting in ➔ increasing the solubility of protein in water till a limit when all water molecules is occupied by proteins and the concentration of Salt is more then it start to decrease the solubility of protein to Water .

SALTING OUT: With increasing amount of added salt, salt ions exceed the desirable level – the concentration Becomes higher than required- and so those ions to start to bind water more effectively than proteins because they are small molecules, and so the amount of water available to bind with protein is decreased, and so proteins will start to precipitate. Once it’s precipitated, it starts forming bonds among itself, but not by the hydrophilic amino acids(a.a) as they no longer have water to be soluble in, so hydrophobic a.a will make hydrophobic interactions and so bonds with another molecule of the protein, and so they aggregate together.(Precipitating proteins out of water by increasing the amount of salt)

So the characteristics of this method:

- Depends on solubility
- Most common reagent: ammonium sulphate.

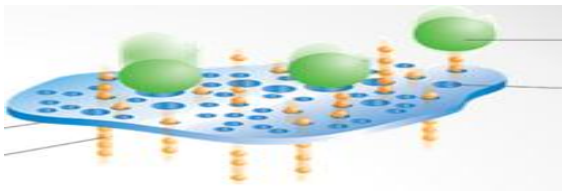
• Nonspecific precipitates all proteins in the mixture (crude)



Dialysis

*It depend on Diffusion theory(molecules eventually reach equilibrium in the fluid) .Every dialysis such as kidney dialysis . we remove small particles while the large particles will be retained in the solution . in kidney dialysis, we remove the toxic small molecules like urea while the large molecules like proteins will be retained back to patient's circulation. It is non specific, you can eliminate some proteins out if they have low molecular weights but you can't select proteins by it (crude)

* Experiment. We get a dialysis bag with 2 sides we fill it with protein mix found in the fluid and tie it from the both sides. The bag contains pores every pore is manufactured for specific molecular weight for example 40 K Dalton .so if we get a protein of 40 K Dalton or any other contaminant less than 40 K Dalton will diffuse. but if we got molecules have dimensions significantly greater than the pore diameter they will be retained inside the dialysis bag (ba7lago) …we know very well from Bio lab that molecules diffuse to outside Cause the concentration is higher inside. So which can't move outside it 'will be retained to dialysis bag (now after . This occurred according to simple diffusion theory. We put a solution (Buffer) inside a tank , beaker or (tanjara) by the time the equilibrium between the inside of the container and the mix inside the bag will be occurred. That's recommend a constant periodic movement of water (or buffer) . How that's occurred? A magnetic stirrer is used with a magnet base to keep it moving and so speeding up the process. How ?As the molecules diffuse through pores they're going to get concentrated around the bag and so prevent more molecules from diffusing outwards, but according to the diffusion principle molecules must diffuse equal to all parts of the fluid and to reach that without a stirrer will take a lot of time (slow process). However, the presence of the stirrer results in constant motion of water and therefore an equal distribution of molecules throughout the whole fluid(equal conc.). . The result of this process is crude. we use it mainly just reduce contaminants and we can remove the proteins which can diffuse through the pores of dialysis bag.



Column chromatography

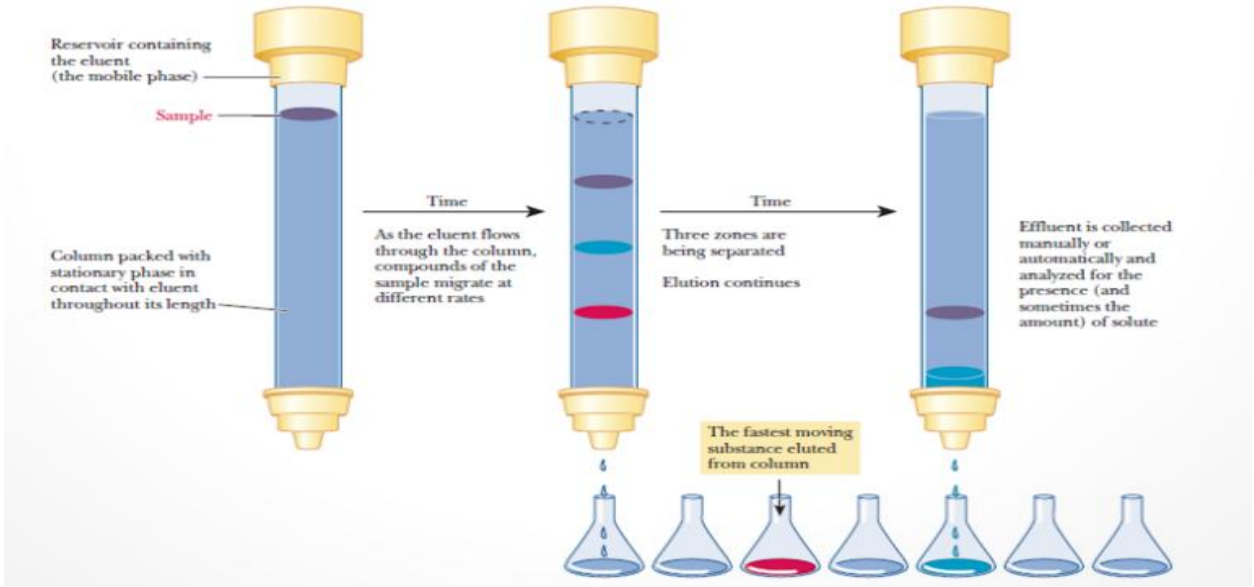
In the past all studies were about colored proteins so all proteins studied were coloured. Chroma = color, Chromatography: we take the color reading as comprehensive result. Column (3amood) .

***Chromatography is based on two phases: stationary & mobile**

1) Stationary phase (static) medium not moving.
2) 2) Mobile phase: Moving phase

**The Overall Idea: we put a material inside a tube the water flow out, so the material will be packed ➔here it is the fixed material and we call it stationary phase a packed material with a high density. Then we put a sample over the stationary phases the sample is fixed on the top till now .afterward, we flow into it the mobile phase (water or buffer {fluid}) then the sample start to move through the column. (Because of the water or buffer movement above the stationary phase). If it is colored it will move till it go out of the column .if it is colorless it also moving till it'll be eluted out of the column but note here we want a way to detect it because we can't visualize it

***According to charge shape Or molecular weight of any protein you have**, movement can be different .so that, the types of chromatography differ. E.g. there is a chromatography just depend on the charge. Other on charge ….etc



What are the different kinds?

A) Size-exclusion chromatography {(Gel filtration Chromatography),(Molecular-sieve Chromatography all are the same but different naming}
B) Affinity chromatography (the Most efficient)
C) Ion-exchange chromatography

A) Size exclusion chromatography

It means you can purify proteins according to their Molecular weight. • (Separation on the basis of size (MW))

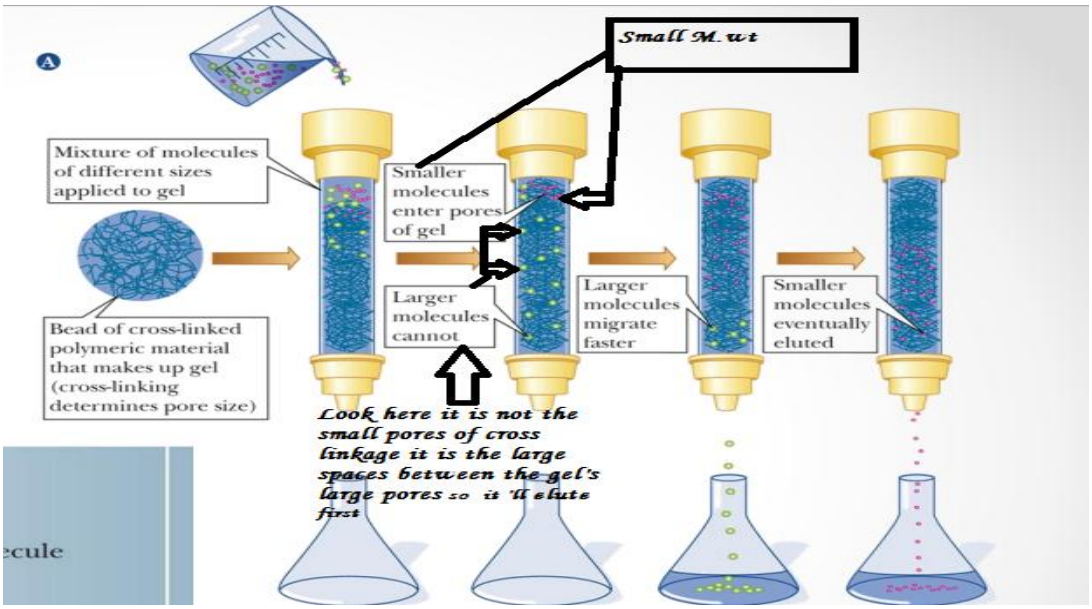
*Gel filtration (the other name): the stationary phase is jelly material and you filtering this protein this material.

D) * Molecular-sieve chromatography : the Idea of Jell filtration Chromatography is just like the idea of sieve.

So In the stationary phase: we fill the tube with mixture of molecules of different sizes applied to a gel (the cross linking determines the pore size) so it is like a circular sieves above each other from the opening till the end .(the sieve Could be(Agaros or Polyacrylamide) which can make cross linking between each other (which determines the pores) the pores of this sieve is bigger than the lines surrounding the pores (as the all sieves in the life) , that helps by which the protein of low molecular weight can places over the lines of the sieve while that one's of high molecular weight couldn't be present over them because of their high molecular weight ,so they will fall in the pores and elute firstly. According to this procedure the proteins of High molecular weight (M.wt) will fall in the pores and elute first (refer to illustration to know which pores but in lecture we took it generally by the term pores so never get missed if u look to the illustration and found (not cross the pores , they meant by that the pores of cross linkages . in fact, the high m.wt move through the spaces between the gel stationary phase not the same pores) ^_^ .but that ones of low (m.wt) will be placed over the lines of the sieve, it recommend water to move it and then fall in the pores of cross linkage ^_^)

Stationary (cross-linked gel particles): consist of one of two kinds of polymers; the 1stis a carb. polymer (ex. Dextran{{can be made by breaking the starch which result in dextran maltose and glucose}} Or agarose); often referred to by Sephadex and Sepharose. The 2ndis based on Polyacrylamide (Bio-Gel)• Extent of crosslinking & pore size (exclusion limit)

• Convenient, MW estimate and it I specific but it doesn't give pure protein)



(Sieve chromatography)

*Affinity chromatography:

Separating the protein according its affinity to some material. This is the most efficient in making pure proteins (the best).

Stationary phase here (whatever what is it crabs ,agaros ,polyacrylamide …etc) we bind it with ligend covalently (remember wa2t eddctor masak esslek t3a al projector section 1) the ligand can bind with a protein of interest (you want only) .For example : the ligand could be an antigen and you want to make a purification for a specific antibody ➔so you spell the sample on top of it ➔ the ligand do not bind except for its specific antibody➔everything moving inside the column will elute outside but (except) the protein of antibody which already bonded to the ligand .as we said before the bond between the stationary phase and the ligand is covalent . While the binding between the protein of interest and the ligand is non-covalent.

The ligand could be : antibody and you are adding antigen ,antigen and you are adding antibody .Ni and add the protein contained 6 histidine (His tags) the Ni have a high affinity to histidine so any protein posses 6 histidines it 'll bind to it . Ni is the ligands so it is the one bonded to the stationary phase. GST-Tag(the enzyme is boned to its substrate but here with more further reaction (ligand the glutathione and the GST its enzyme .

Result of this procedure is very pure.

How can I breakdown the covalently linkage between the protein and the stationary phase? if you add the same ligand in big amounts in the solutions they will compete with the upon the bond with the protein. On a specific concentration they will all surround the protein and get it out eluted (the protein of interest is eluted with its ligand) ➔how to remove the ligand? > Centrifuge it ,so all the water will be out(making it dry). Then adding a buffer.Refer to illustration in the slide (change in pH or ionic strength)

*Ion exchange chromatography:

*****it depends on ions. The stationary phase (Resin) itself could be either positive or negative exhibits an overall net charge at given pH.. Every protein consists of AAs.(either +vely Or negatively charge amino acids . we know we can calculate the isoelectric point of AA and it is applied for protein . if the protein carry a +ve charge it will bind to the -ve Resin like a caroxymethyl cellulose) .how this occurs ? when we put the sample containing the protein of interest it will bind to a risen of the other charge by a non covalent bond but the protein with the same charge will elute at the beginning .other risen DEAE (diethylaminoethyl)which carries a +ve charge.

* Most of proteins have net +ve charge.

* if the protein bonded to the Risen (stationary phase) (+ve with Negative) how can we breakdown the linkage ?1st by changing the pH . the pH will change the charges of AAs then it will break the salt bridges between the (+vely charged and negatively charges amino acid) resulting in breaking the bond between the protein and the stationary phase .2nd adding salt so the salt will compete with protein to bind to the stationary phase . Once you increase the concentration of the salt u increase the competition

** Negatively charged proteins can be separated by a positively charge coloumns , such as diethylaminoethyl-cellulose coloumn (risen) this is known as anionic exchange chromatography.

**this is mentioned in the slides but prof did'nt say anything about ,positively charge protein binds to negatively charged column this positively charged protein will elute after increasing the concentration of salt e.g (NaCl) or other salt in the buffer because the positievely charged molecules will compete with +vely charged group in protein for binding to the coloumn .here protein with net positive charge will elute firstly followed by high density positive charged proteins and this is called cationic – exchange chromatography.

**Electrophoresis or (gel electrophoresis):

The motion of protein with a net charge through the gel pores (which Serves as a molecular sieve) under the influence of electrical field according to shape, charge and molecular weight.

*Used to estimate the protein you have through it is molecular weight so it offer strong mean of separating proteins

*The material the gel is made up from are Agarose and polyacrylamide. agarose is better for DNA because of the small pores inside .while, polyacryalmide is used for proteins since it got a large pores made up by cross linkage.

*If the protein is denatured, (in the indentured protein, the charge of it is determined through the AAs at the surface .but once we denature it . the AAs are become liner and they are all share in forming the overall charge of the protein (cause it is Linear Now .remember :denaturation is loosing of tertiary structure and resulting in retaining the 1ry linear structure of protein or DNA) .Now, All Amino Acids are exposed to the solution .so now the charge may be increased or decreased so it is not useful in differentiating protein , even through the shape hence after denaturation all proteins become linear.

* The only protein determinant property when it is denatured is the Molecular weight.** Ladders: they 're set of standards that are used to identify the approximate size of a molecule run on the gel

with the protein of the interest under the influence of electrical current and gives different bands . Bands: pieces of protein with specific Molecular weight.

*The most commonly used protein electrophoreses technique is termed SDS Polyacryamide gel electrophoresis (SDS-Page) it works as a denaturing gel so the protein must be move under the effect of molecular weight. If we put a material which is not denaturing (native gel a term for non denaturing) gel so the protein will move under the effect of charge shape and molecular weight. You can't determine the m.wt using a native gel.

* if we put a hemoglobin or myoglobin in a denaturing gel which are consist of many subunits hemo 4(2-alpha 2-Beta) myo (8 alpha helices) < look before denaturation . Now, their movement in the gel will be totally different than the protein of one subunit as they are consisting of multi subunits. But when we make a deanturaton for them using the SDS we retain the primary structure by separating the subunits and retaining the linear structure ((Alfa will move together , Beta will move together)

* SDS (Sodium Dodecyl sulfate) gel has a uniform negative charge on it. What is the work of negative charge?

--it denatures and solubilizes protein

**Agarose vs. PAGE

- We use agarose to clarify DNA , but polyacrylamide gel (PAGE) with proteins…why ?!

*Because the agarose pores are smaller than PAGE ones …. But still we can use agarose with very small proteins or peptides .

**Isolelectric focusing:

We use an isoelectic focusing gel here. In addition , we can use the polyacrylamide

gel to determine the isoelectric change of protein . we know very well that every protein posses its isoelectric point at certain pH. Here is the polyacrylamide is made with a variant pH levels (gradient)protein ,so how this variant pH degrees obtained , we put several materials making a variant pH levels (doctor didn't say their name but they called ampholyetes).therefore the protein will migrate through the gel till it reach the isoelectric point of its own(in which the total charge of protein will be zero) .once it reaches this point when pH equal to pI of protein it 'll stop moving . this is a way also to identify the isoelectric point of the protein . so when the protein move under the influence of electric current till it reach the point (when pH is matches) previously mentioned it 'll stop moving.

*Two dimensional gel electrophoresis (genius techniques) ((2d-Page)

**Combining 2 techniques together how??

---firstly we apply isoelectric focusing techniques by which the protein will stop at the appropriate area of pH (the area of isoelectric point of the protein)),then through as SDS-PAGE after rotating the stationery phase 90 degrees . The SDS page will denature the protein . in addition it will give 'em a negative charge through which it denature it. forward , applying electric current . So the protein will move according to its molecular weight (SIZE) ^_^.

Protein sequencing

*It is the process of knowing the amino acids sequence of a protein or a peptide.

*****how can we know the sequence of the protein ? By degradation. The most useful method called Edman method of degradation

*** Edman method:** 1st step: prepare one molecule of protein and keep it boiling at 100-110 and put over it high amount of salt concentration (acid concentration of 6M HCL) add it while heating .by the time all peptide bond present in the molecule will be degraded. Then the result is single amino acids. After that , there is an appliance called Amino Acid Analyzer which can detect them (e.g 20 Tyr, 10 Gly , bla bla..Etc...). Method summary: Hydrolysis (heating + HCl) & Separation

(Ion-exchange chromatography or by high Performance liquid chromatography, HPLC)

- This method (still it is Edman) provides quantitative and qualitative detection for what the amino acids present inside the protein.

2nd steps: we get another molecule of protein and through specific procedure you 'll determine the identities of N- & C- termini ends of it.

3rd step: cleavage into smaller fragments(specific peptide bonds) By adding material which could be :

1) Enzymes- Trypsin, Chymotrypsin.

2) Chemical reagents- Cyanogens bromide CNBr (have the ability of breaking specific peptide bonds).

* The examples for enzymes: you can add trypsin or chymotrypsin both are found in the small intestine.

* So what the work of enzymes or these chemical reagents? They break proteins at specific amino acid (e.g trypsin always cut after positively charged amino acid. so I put a protein of 20 residues AAs ➔ the 20th Amino acid is positively charged one (remember +vely charge AAS are Lys, Arg and His).

*Chymotrypsin always cut after an aromatic containing Amino acid (Tyr, Try and Phe){e.g. protein of 15 AA residues the 15 Must be one of three previously mentioned AAs) .

_*Chemical reagents

Cyanogen Bromide: This reagent always cuts after C-terminal side methionine.

Eg about third step. when we add CNBr(as a chemical reagent it will break down internal methionine from C-terminus end . so every piece from broken from the C- terminus end is Methionine..Then we start adding enzymes, we keep on adding , different results will appear, till we reach the sequence of protein at the end after collecting the several results of enzymatic effect.

- We can also make a "prediction of the protein sequence from the Gene sequence .why? because genetic sequence consist of, codons, they are three-base sequences that specify the addition of a single amino acid
- Now if we don't wanna go through gene sequence and we wanna be away from edman degradation method because it last long and it is costly what should we do???

-- to sequence a short section, (perhaps 15 amino acids long), of the protein, and then use this sequence to generate a complementary marker for the protein's RNA ((By other words : remember Small interfering RNA this is an abstract from sheet #9 : we can see the sequence of bases included in it the M-RNA that is formed to synthesize protein, {{you design a small piece of this RNA to be complementary to the mRNA so after injection in the cell it will bind to the mRNA. if it is bond the M-RNA then it will not be able to go to ribosome and get

Expressed ==> so no protein formed □ u can study effect while no protein is there this procedure is called knock down and by this we know the sequence of amino acid.

In the slide there is a technique written there while the professor didn't tell anything about which is (PCR) this is called polymerase chain reaction .

Immunoassays – Western blot "Protein Immunoblot

*It is named western-blot because of (nagashet ennerdat)

*western blot: it is the acrylamide Gel itself . ** It`s based on Electrophoresis.

* method: In Immunology we can`t detect the protein while being in a Gel… so we are going

to transfer(move) the protein to a membrane often made of nitrocellulose or polyvinylidene

difluoride, PVDF using electricity again from the gel to a Membrane that can Absorb proteins. Then we place this protein in a beaker, afterward, we add antibody specific for the protein You wanna detect it to determine whether it is present or not because we got a mixture of proteins but we want to detect the protein of interest .so after adding the antibody and leaving it for an hour then we wash it and bind it with a secondary antibody , the secondary antibody bonding to the 1ry antibody which is bonded to the protein we want to find it ☺ wow :P . secondary is more specific than the primary giving very specific procedures .

There are four procedures to detect the protein specifically :

1)if the enzyme is bonded to it we can determine the protein specifically , how ? the enzyme will bind to a substrate giving a specific color. If the color doesn`t Change however, then there is no reaction and this means that there is no enzyme, which means that the 1ry antibody didn`t bind to the protein, which means that there was no protein to begin with this method of detection called Colorimetric.

* 2nd: method of detection of actual protein called Chemiluminescent .here the enzyme have a ligand on the secondary antibody illuminting when there is a protein ➔ binding to 1ry ➔ the secondary bonding to it . Illumination occring and can be detected by special camera called CCD cameras

3rd way of detection : here on the surface of the antibody there is a fluorescent material .once you make excitation for it through a certain wave length then we can detect it

4th way : if there is a radioactive material on the secondary antibody :if we take the membrane and make an imaging for it through x-ray we will detect the protein on the membrane .

* ***one more time in other way with revising past info, Why do we use the Western Blot ?To be sure that we have a protein of interest, using the tags (ex: Histags) and binding it to a specific Antibody in order to identify the protein.

ELISA: Enzyme-Linked Immunosorbent Assay

The general Idea : the sample we already got contains antigen or antibody ,then the antigen or antibody will come and bind to other antigen or anti body . then we add secondary antibody to provide specificity . on the enzyme then we will do the same thing of wetern blot method (flurescent material , ligand ,substratre if the protein bonded the colour will change and radioactive) .

Application for ELISA: In pregnancy test : we bring a urine sample ,the urine sample put over a test strip .the test strip contains three areas . 1,2 and 3 . area 2 and 3 contains fixed antibodies by a strip .area 2: contain primary antibodies . area 3 contains secondary antibodies .area 1 contain free primary antibodies (not fixed in its place). Once this course of urine put over the strip it will start move according to diffusion theory ,now we are looking for a the hormone HCG . This protein (hormone)will produce antibody . antigen will bind to the antibody in more than one place . If the urine doesn't contain HCG .the uraine sample will complete its movement according to diffusion. Here is dissolved antibodies in sample and it 'll continue its movement inside the strip. In pregnancy the primary antibody binds with 2 Gondtotropin hormone so the 1ty antibody will be sandwiched between both 1ry antibodies. The primary bind also with secondary antibody result in 2 colours . while in no pregnancy . the primery antibody cant bind with gonadotropin but, even will not bind to a 1ry antibody and it will bind to secondary anti-body resulting in a color .

There are 2 types of ELISA…

1) Coat plate with Antigen: used for Screening (HIV, Hepatitis B&C).

2) Coat plate with Antibodies: used for Hormones (HCG, LH, TSH, T3, and T4).

Past papers question concerning the subject : ((hint they are 3 of them directly related to the subject while 2 not directly related to the subject :

1) Regarding the pI of the protein which of the following is not correct :-

A) The pI is the pH which a protein has total net charge of 0

B) A basic protein will have a pI greater than 7

C) The pI is the pH at which a protein has no negative or +ve charges

D) A protein has least solubility at pH equal to its pI

2) a mixture of proteins us applied in a pH 6.5 buffer to an anion –exchanger chromatography coloumn and eluted. What is the first protein to be eluted

Protein	pI	Mol wt
urease	5.1	482,700
catalase	5.6	247,500
Lactoglobin	5.2	37,100
hemoglobin	6.9	64,500

A) Urase B) lactoglobin C-Catalase D-Hemoglobin

E-Both hemoglobin and lactoglobin

3)Which of the following can make protein denaturation :

A) 6M giandine B) 70M ethanol 3) 8M urea 4) strong acids or bases

4)the major role of SDS –Page is to

A-Make proteins negatively charged

B-Increase buffering capacity C-prevent proteins from denaturation

D-Increase the pI of proteins E- prevent proteins from degradation