CHAPTER 1

1. INTRODUCTION:

1.2 History of discovery of transglutaminase

Transglutaminase is a group of enzymes in which played an important role to catalyze the formation of non-disulfide cross linking bonds between glutamine residues and amine groups (Klock et al, 2014). Furthermore, transglutaminase is capable of catalyzing the calcium-dependent bonds (Piper et al., 2002). This catalytic activity is required calcium which is responsible for inducing the conformational changes in the enzyme (Stamnaes et al, 2010). Historically, in 1950, a researcher has been discovered this group of enzymes and the word of transglutaminase, when he was studying the enzymatic activity of the liver extract from the guinea pig (Fesus and Piacentini, 2002). Later on, Harding and Rogers identified a hair protein extract that formed cross-links similar to that of transglutaminase 2. They found out that there were three enzymes that were expressed both in the hair follicle and keratinocytes to avoid confusion; they named the Iso-enzymes based on their genes. Eventually, other enzymes belonging to this group were discovered through sequence homology (Odii, 2014). The enzymes catalyzed the incorporation of a primary amine into proteins a process known as deamidation that requires calcium ions as a cofactor (Odii and Coussons, 2014). The transglutaminase family consists of nine enzymes with the transglutaminase 2 enzyme being the widely studied enzyme. The enzyme is also called the liver or tissue transglutaminase (Pinkas et al., 2007).

1.3 Family of Transglutaminase 2:

The family of transglutaminase 2 consists of nine enzymes whose mode of action is similar and their structure and function are related to each other. The enzymes are expressed in different tissues with different substrates. The enzymes include TG1, TG3 and TG5, which are all isoforms and found in the epithelial tissue. The molecular weight of TG1 is 106 KDa while in TG3 is 77 KDa. In addition, the molecular weight of TG4 is 80 KDa which is found in the prostate gland, TG6 in the testis, lungs and brain (Thomas H et al, 2013). Factor XIII and FXIII are expressed in the blood while TG7 is expressed in all tissues but acts on testis and lungs (Benedict and peter, 2014). Band 4.2 or FXIII is a member of TG2 family and enzymatically is inactive protein which shared the homology with different transgutaminases. At the active site of the enzyme, the amino acid is replaced (Cys-Ala). Therefore, it lost the characteristic of the transglutaminase activity (Lorand L and Graham RM., 2003). TG2 is the widely studied enzyme that is expressed in the liver. TG2 has several isoforms that are due to alternative splicing of the transglutaminase 2 encoding gene (Citron et al, 2002). TG2 is responsible for the post-translational modification in proteins that results from the cross-linking activity of the enzyme. The reaction is calcium dependant and involves deamination and deamidation process (Odii, 2014).

1.4 The structure of TG2

The fundamental structure of TG2 is similar to the other transglutaminase family enzymes. Tissue transglutaminase has a complex structure that is 78-kDa. The complexity of the enzyme has hampered effort to study its mode of action. However, in its open structure it has active sites that have been examined to reveal the mode of action of the enzyme. The enzyme

undergoes to some conformational changes once the substrate binds to the active sites as compared to the unbound enzyme (Pinkas et al., 2007). The enzyme is in the form of GDP in its closed form. The amino group of lysine crosslink with the carboxyl groups of other amino acids forming bonds that are resistant to degradation by proteases. The enzyme has four domains namely NH2-terminal β-sandwich that includes integrin binding sites and fibronectin. The catalytic core that functions as acetyl transferase during enzymatic reactions and two C-terminal β-barrel domains (Mehta and Ecker, 2005). There is a unique binding site which located between the first β-barrel and catalytic core that is called guanidine nucleotide-binding site.

Moreover, the phospholipase C binding sequence is located on the second barrel domain (Mehta and Ecker, 2005). The active site is composed of cysteine proteases that function in a transamidation process. The cysteine proteases have the following functional groups cysteine 277, histidine 335, aspartate 358 which takes part during transamidation process (Soner et al., 2012).

1.5 The activity of TG2

Tissue transglutaminase is a calcium dependent enzyme found in the intracellular and extracellular matrix of the cell (Odii and Coussons, 2014). Transamidation is the process by which amines are incorporated into proteins. The process takes place in two steps. In the first step, nucleophilic attack on the Y-carbon of the glutamine side chain to form a thioester bond the active site residue and the substrate (Soner et al., 2012). The active residue in the active site is cysteine 277. Ammonium molecule is released as a by-product. In the second step, the thioester bond formed in the first step is broken by an amine or water. In the event that the primary amine attacked the thioester bond, the reaction is termed as transamidation while if the water molecule

did the attack, the process is known as deamination (Soner et al., 2012). The addition of an amine group to the protein has some biological meaning. The addition results in post-translational modification of a protein that changes the conformation and the function of the resulting protein. Deamination occurs mainly in the absence of primary amines or when favored by conditions such as pH and temperature (Odii and Coussons, 2014). Certain studies have shown that TG2 and G protein are the same. Ca^{2+} and GTP binds to the enzyme making it to operate like the G protein. However, the physiologic role of TG2 acting as a G-protein is still not well understood due to the presence of contradicting results (Soner et al., 2012). Moreover, apart from calcium dependent functions the enzyme has other duties that are independent of calcium ions. Tissue transglutaminase also functions as enzyme kinase. It is involved in the phosphorylation of proteins which results in a functional protein. It is a form of post-translational modification that occurs in the nucleus after translation of DNA. Finally, the enzyme can act as a protein disulphide isomerase. A study showed that TG2 converted inactive RNase into an active enzyme by forming the disulphide bonds (Soner et al., 2012). The process is calcium independent and the active sites involved in the deamination do not participate in this process. The activity of TG2 has been shown to play a crucial role in celiac disease and other diseases (Klock et al, 2012).

1.6 Transglutaminase 2 in Cell Survival and Death Processes

Transglutaminase 2 acts as a pro-apoptotic agent both in the embryo and in adult life. In transformed cells, the enzyme achieves a dual role, as both a pro-apoptotic and an anti-apoptotic agent. These have been observed in the same type of tumor. This switch has been suggested to be dependent on environment and genetics (Toone, 2011). The role of TG-2 in the process of cell

death has been determined to depend on the sub-cellular localization and cell type stimuli, and the state of conformity of the protein (Gundemir et al, 2012).

1.6.1 Pro-apoptotic Activity of theTG2

The role of TG-2 in the process of cell death has been determined to depend on the cell, stimulus, intracellular accumulation and the stereochemistry of the protein (Gundemir et al, 2012). Ethanol induces apoptosis in hepatic cells by increasing the expression of TG2 and enhancing its activity. TG2 inturn causes formation of linkages and inactivation of Sp1 that subsequently inhibits c-Met that is crucial for liver cell survival and normal function (Tatsukawa et al., 2009). TG2 acts as a signal partner in stabilizing the bond between β3-integrin/ protein milk fat globule EGF factor complex. This complex is integral to the upregulation of RhoG and Rac1 signals that acts to increase apoptotic and phagocytic signaling (Eckert et al., 2014). The activation of TG2 causes formation of cross-linked protein structures typically observed in apoptotic cells.

1.6.2 Anti-apoptotic Activity of TG2

A high TG-2 concentration leads to increased invasiveness and tumor cell survival (Chhabra, Verma & Mehta, 2009).The EGFR pathway is activated in malignancies caused by up-regulation of TG 2 production in cervical and epithelial cells in breast cancer. This pathway combined with another known as JNK/ERK signaling pathway have been found to mediate inhibition of Tumor Necrosis Factor-related Apoptosis Inducing ligand(TRAIL) and invasiveness (Nurminskaya & Belkin, 2012). The expression of Hypoxia Inducible Factor-1(HIF-1) also confers resistance to apoptosis through exposure to hypoxia. The mechanism of this process is the expression of TG2

activated by a HIF-1 reliant pathway (Jang et al., 2010). The TG2 produced in this way suppresses apoptosis by modulating the NF- κB and caspase-3 roles in activating apoptosis in hypoxic tumor cells (Kim et al., 2006). TG2 down-regulation by small interfering RNA among other inhibitors has been shown to amplify the vulnerability of cancerous cells to chemotherapy induces apoptosis. This suggests that TG2 is a major factor in the invasiveness, drug-resistance and increased survivability of tumor cells (Onyekachi, Ebidor & Maxwell, 2015).

1.7 Role of tissue transglutaminase in various diseases

1.7.1 Neurodegenerative disorder

Various neurological conditions have been implicated by the cross-linking ability of TG2. Posttranslational modifications of proteins brought about by the enzyme during transamidation changes the function of the proteins. These modifications cause significant effects on the nervous system. Recent evidence shows that tissue transglutaminase synthesis is controlled by the nervous system. The intracellular neuro fibre tangles have been aggregated with amyloid Beta-protein and produce a highly phosphorylated type of protein that allow the Alzheimer's disease to be characterized into various types (Hoffner & Djian, 2005). Up-regulation of the enzyme results in the etiology of neurological disorders such as Alzheimer's and Huntington's disease (Martin et al, 2011). The substrates of the enzyme after deamination are responsible for the neurodegeneration resulting in conditions like Parkinson's disease. The metabolites include synapsin 1and myelin basic protein.

1.7.2 Role of Tranglutaminase 2 in Cancer

Cancer is the uncontrolled proliferation of cells. Transglutaminase 2 plays a significant role in the contribution of uncontrolled proliferation of cells. Certain researches have been demonstrated that there is a positive correlation with TG2 expression levels between the metastatic potential of certain cancers and chemotherapeutic resistance (Mehta et al, 2004). Moreover, the previous studies proved that the amount of the TG2 is increased in ductal adenocarcinomas, glioblastoma and pancreatic malignant melanomas in cancerous tissues (Tucholski et al, 2006). The enzyme possesses a cross-linking activity of proteins that is responsible for the pathogenesis and etiology of the cancer. The process results in modification of adhesive cells, their migration and protein expression leading to uncontrolled proliferation (Lin et al., 2015). Polymerization formed by cross-linking has been shown to increase drug resistance in cancer cells. Cancer cells have higher expression of the enzyme, that help the cells in metastasis. And mesenchymal phenotype that aids in the invasion of cancer cells (Jones et al, 2006). Transglutaminase2 has been exerted to the anti-apoptotic effects in certain types of cell whilst these cells sensitize to apoptosis during TG2 inhibitors treatment or siRNA down-regulation of TG2 (Yuan et al, 2005). Other studies suggested that TG2 plays a significant role as a protein that suppressing the tumor through binding and interacting with GPR56 (Xu et al, 2006). Thus, the role of TG2 to cancer might be dependent upon the stage of the cancer, the location of the cancer, the type of cancer and cancer cell type.

1.7.3 Transglutaminase 2 inhibitor

The mechanism of inhibition of TG2 plays a crucial role to divide the transglutaminase 2 inhibitors into three different classes which includes competitive inhibitors, reversible inhibitors and irreversible inhibitors. Firstly, competitive inhibitors are the most common utilized TG2

inhibitors, the main reason of using that because they have certain properties. For instance, in living systems are non-toxic, commercially available and chemically stable (Karpuj et al, 2002). Cystamine has numerous inhibition mechanisms such as diamination and that make it as a unique inhibitor of TG2 (Jeitner et al, 2005). Secondly, without any changes in the covalently of enzymes for instance, GTP and GDP, reversible inhibitors are blocked the substrate gate of the active site and that leads to prevent the enzyme activation. Thirdly, by modifying the enzyme covalent, irreversible inhibitors are prevented the activity of the enzyme. Therefore, it prevents the substrate binding.

1.7.4 Role of Transglutaminase in Fibrosis

Fibrosis is a condition that leads to the deposition of fibrous substance in the organs causing pain. The most affected tissues include heart, kidney, liver, lungs, skin and the blood vessels. In addition, fibrosis is characterized by accumulation extracellular matrix proteins and fibroblast (Olsen et al., 2011). The accumulation impairs the normal structure and functioning of the organs. Histological assessment of a diseased organ reveals the presence of myofibroblasts, contractile cells and smooth muscle cells (Olsen et al., 2011). Tissue transglutaminase has been demonstrated as a possible etiology of the condition whereby it crosslink's irreversible with extracellular matrix or else acts by recruiting transforming growth factors to these organs (Tim et al., 2007). Myofibroblast are important cells in the formation and progression of the fibrosis. They secrete extracellular matrix components such as collagen and fibronectin that upon cross-linking forms the fibrosis. TG2 is almost found in all cell types in both extracellular and intracellular. In high concentration of calcium ions in the extracellular matrix, the enzyme is activated leading to cross-linkage of collagen and fibronectin (Olsen et al., 2011). The matrix

formed is resistant to breakdown by plasmin. TG2 in the cytosol affects the cell cycle since it functions as a G-protein. It binds to the integrins on the cell surface affecting the cell adhesion and mobility (Olsen et al., 2011). Cell adhesion and mobility are characteristics of cells that are enhanced by the TG2 independent of its enzymatic functions, interestingly over expression of the enzyme affects these characteristics and predisposes the cells to fibrillation (Wynn, 2008). Inhibitors of tissue transglutaminase are used in the treatment of fibrosis (Shekwe et al, 2009).

1.7.5 Role of Transglutaminase 2 in Ciliac Disease

Celiac disease is an autoimmune disease brought up by the production of antibodies that recognize self-proteins as non-self (Giersiepen et al, 2012). The higher production of TG2 antibodies is associated with disease and that suggests the role of TG2 in celiac disease (Klock et al, 2012). Celiac disease has been associated with certain symptoms such as anaemia, diarrhea, pain, chronic constipation, failure to thrive (in children). In addition, the disease can be either caused by genetic predisposition or induced by gliadin peptide which is a toxic component of the gluten. After gluten ingestion, tissue transglutaminase forms a cross-link that leads to formation of proteins that elicits the immunological response towards the proteins (Fantana et al, 2011). The immunological response activated may be innate or adaptive immunity. Deamination of glutamine in gluten proteins have been shown to cause the toxicity that results in the formation of celiac disease (Ciccocioppo et al, 2005). The disease mostly affects the gastro intestinal tract by making the intestines leak enabling the contents in the intestines to enter the blood stream. Antibodies against gluten enter the system confusing tissue transglutaminase to gluten. This results to an autoimmune disease where self-antibodies attack self-proteins. Stomach pains are frequently experienced especially on ingestion of foods rich in gluten proteins (Hunt et al, 2008).

These have been hypothesized that TG2 plays a crucial role to contribute to the pathophysiology of the disease.

Prolamine is a protein that is expressed in barley, wheat and rye and is the main responsible to cause the inflammation in celiac sprue and small intestine (Sollid, 2000). During ingestion, the high content of the glutamine and proline proteins can be resistance to natural intestinal proteases and peptidase and also resistant to natural pancreatic and gastric proteases and peptidase (Shan et al., 2002). Deamidation of peptide is caused by TG2 and it has been shown to increases the severity of disease through the excellent correlation between T cell stimulatory potential of TG2 which treated by prolamins, DQ2 binding affinity and TG2 substrate specificity (Qiao et al., 2005). A research suggested that the inhibitors of TG2 have a major role to decrease the influence of celiac disease (Siegel and Khosla, 2007).

1.8 Structure of gluten and gliadin

Gluten is a protein which found in cereals especially in wheat. It is not a single protein but rather is a combination of proteins formed when flour is mixed during baking (Catassi and Fasano, 2010). In the gut, gluten is broken down by enzyme tissue transglutaminase produced by the intestinal walls to form gliadin and glutenin (Koskinenetal et al, 2010). Glutenin is alcohol soluble protein that is resistant to digestion by protease enzymes in the gut leading to its accumulation in the gut (Catassi and Fasano, 2010). Gliadin peptide (zonulin) is involved in receptor binding which in turn leads to the opening of the intracellular tight junctions. The binding mimics the action of immunological functional modulators of the tight junction (Fanaso, 2011). The accumulation of gliadin in the lumen makes the gut leaky allowing components of the

digestive system to enter the blood stream. In the epithelial cells, the peptides interact with cells of the lamina propria that function as antigen presenting cells. Lamina propria cells, on binding to the peptide promote an inflammatory response responsible for the symptoms presented by celiac disease (Koskinen et al, 2010). The process involves both innate and adaptive immunity to produce inflammatory cytokines. Deamidated gluten is whereby water molecules attack the thioester bond. It is a form of posttranslational modification where the new protein has a different role (Pinkas et al., 2007). The disease is responsible for the destruction of the intestinal mucosa hindering the absorption and digestive process of the intestine. The symptoms of the disease include stomach clumps and pains, diarrhea, gastrointestinal motility and vomiting. The only therapy that has been found effective is nutritional therapy where gluten protein is completely cleared from their system. Patients are advised to avoid diets rich in gluten protein (Myers, 2013). A study established that gluten-free diet plays an important role in the improvement of outcome of antibody response and intestinal mucosal inflammation (Klock et el, 2012). In addition, TG2 inhibitors have been used as a potential treatment for celiac disease and considering that the protein is associated with the pathophysiology of the disease (Klock et el, 2012). Serology testing has been widely used in diagnosis of the disease. Antibodies against tissue transglutaminase are used to detect the availability of the enzyme. They also target the deamidated gliadin peptides (Giersiepen et al, 2012). Detection of IgA antibodies produced towards the enzyme is sensitive and specific. The test targets the auto-antibodies produced against the self-proteins (Giersiepen et al, 2012). The method is cheap since it uses enzyme linked immune assays for detection. Finally an intestinal biopsy can be done by the doctor to reveal the damage of the intestine (Hunt et al, 2008).

1.9 AIMS OF PROJECT

The main aims of the project are to partial purify TG2 from gpl, develop TG2 enzyme assay by using biotin cadaverine that utilized to determine the activity of TG2 inhibitors. Develop florescence assay that utilized to detect the deamination activity of TG2. And also develop an electrophoretic assay for TG2 deamidation activity to show the inhibition by standard TG2, inhibitors such as polyamines, Z-DON, R283, EDTA and ca^{++}.

CHAPTER: 2

MATERIAL AND METHOD:

2.1 Protein assay

Protein assay is a technique that utilized to quantify and determine the concentration of protein in sample. This procedure was carried out according to the material and methods of smith et al (1985). To carry out a protein assay you require the following stock reagents:

1. Stock reagents A: 50 %w/v sodium hydroxide, 0.16%w/v sodium tartrate, 2%w/v sodium carbonate, 0.95% sodium bicarbonate, 0.4% w/v sodium hydroxide and 1%w/v BCA-Na2. (Sigma aldrich-life science).
2. Stock reagent B: 4% w/v copper sulphate ($CuSO_4 \cdot 5H_2O$). Both reagents A and B ought to be stored at room temperature to avoid deterioration.
3. Reagent c: mix reagent A to reagent B at the ratio of 50:1. The mixture formed is green in colour and stable at room temperature for one week.

Pipette 250 µl of reagent C and add to 25 µl sample containing 2.5–30µg of the protein. Incubate the sample for 30 minutes at 37 ºC and measure the absorbance at 562nm. The plotted graph will help determine the protein concentration.

Protein concentration (mg ml^{-1})	Vol (ul) of BSA stock (1.0mg ml^{-1})	Vol of buffer (µl)	Vol of reagent C to add (µl)
0	0	25	250
0.1	2.5	22.5	250
0.2	5	20	250
0.4	10	15	250
0.6	15	10	250
0.8	20	5	250
1.0	25	0	250

Table shows the protein standard of BCA

2.2 Extraction of Transglutaminase 2 from guinea pig liver:

To extract TG2 from gpl, chop the gpl into small pieces and add liquid N_2. Liquid nitrogen will aid in grinding it until it becomes powder then add 10 ml of buffer per gram of tissue. Centrifuge the resulting solution at the rate 12000g for 30 minutes to separate different components in the solution. Carefully take 1ml of the supernatant using micropipette and put them in a freezer. Take the remaining portion, and divide it in 100 ml portions. In each portion of the supernatant add 76g of ammonium sulphate and label the tubes A and B. Centrifuge the resulting solution at 12000g for 30 minutes. Take cellulose acetate tubes and put it in a beaker with boiling water for five minutes after which you discard the water. Repeat the boiling for five minutes. Take the samples A and B from the centrifuge and put them into the cellulose tubes A and B respectively. Take the tubes with the sample and place them in a 5liter container with distilled water for one hour. Remove the tubes and discard the water. Fill the container with TRIS buffer and leave it overnight. Then remove the tubes from the buffer and cut the rubber tubes. Put the solution inside two blue tubes labeled A and B. Top up with water to make the solutions equal and centrifuge at 15000g for 30 minutes.

2.3 Extraction of storage proteins from vicia faba

Take the vicia faba seed and separate the embryo from the cotyledons. Throw away the embryo and keep the cotyledons in a warming beaker. Take the cotyledons after warming and homogenize it in 0.1M TRIS buffer at a pH of 8.5 for 30- 40 seconds. Then centrifuge the resulting solution at 12000g for 30 minutes. After centrifugation, carefully take the supernatant and discard the residue. Prepare dialysis tubes of 4 lengths and half fill the dialysis tube with the supernatant. Boil the tubes in distilled water mixed with EDTA and sodium carbonate for five minutes. Allow the liquid to cool then discard it. Put the tubes now in distilled water and boil for

another five minutes. After boiling, let the water to cool and then pour it out. Tie a knot at the one end and half fill the tube with liquid. Ensure that the tubes are air free by using two fingers and then tie a knot the other end. Put it in a large volume of distilled water at 4 ºC, changing the water at every two to three hours in a cold room until the precipitate forms and leave it for two to three days. When the precipitation is formed, open the dialysis bag and put the liquid into centrifuge tubes and centrifuge it at 12000g for 30 min. After the centrifugation discard the supernatant and keep the precipitated form. Divide the precipitate into two different tubes and wash with the minimum water and put it in a freezer before freeze drying.

2.4 SDS-PAGE electrophoresis

2.4.1 Material

Laboratory Equipment
An electrophoresis chamber
power supply
100- 10 µl pipette
SDS buffer
BIO-RAD 7.5% gel
Water bath
Reagents
100 µl of zedira TG2

100 µl of gpl TG2
Standard protein marker
Sample buffer (Laemmli sample)

SDS-PAGE Equipmen *SDS-PAGE Reagents*

2.4.2 Sample Preparation

Take 100 µl of zedira and 100 µl of gpl and after that add equal volume of Laemmli sample buffer to each protein, then mix and boil them at 95 °C for 5 minutes. Leave the samples at room temperature and then start loading.

2.4.3 Electrophoresis

Electrophoresis is the process whereby the proteins are separated based on their size. The technique relies on the fact that proteins are negatively charged hence will be attracted to the positive electrode. SDS is a buffer that covers the protein molecules giving it a net negative charge. Gel forms a meshwork with tiny poles that hinder the movement of proteins from the negative electrode to the positive electrode. Small sized proteins will move faster than the bigger ones hence the separation. This procedure has been done according to laemmli (1970). SDS-PAGE.

2.4.4 Method

Prepare a mini protein TGF precast gel from Bio-Rad at 7.5%. Remove the green sticker which is found at the lower end of the gel and remove the comb from the upper part of the gel. Wash

the gel with distilled water to remove traces of methanol and unploymerised acrylamide. Then take the gel into the electrophoresis tank and add enough SDS buffers to cover the wells and the outside area of the flame. Load the samples using a micro pipette into the wells and cover the electrophoresis with a lid. Assemble the electrodes and adjust the current and allow the proteins to separate. Run the gel at 40 mAmps for each gel and leave it running for an hour. After the protein has separated, withdraw the power, the gel is ready for further procedures.

2.5 Western Blot

Western blotting is a technique used to determine specific proteins after gel electrophoresis. The negatively charged proteins are immobilized on a positively charged matrix where identification of the protein is done. Western Blotting was utilized according to Towbin et al (1979).

2.5.1 Materials

Laboratory Equipment	**Reagents**
Blotting apparatus	3% (w/v)of milk protein (marvel)
nitrocellulose membrane	PBS Tween
nitrocellulose membrane filter	Primary antibody
Gel roller	Secondary antibody
Tissue paper	ECL
Forceps	
Shaker	

Western blot Equipment *Western blot Reagents*

2.5.2 Method

After running the gel at 40mAmps per gel and 200 V for one hour, remove the gel from the cassette and divide it by two. The first half is to be used for protein detection whereby the gel is stained with coomassie blue (Instant blue, Expedeon Cambridgeshire, UK) to improve the visibility.

The remaining part of the gel is used in western blotting whereby the proteins in the gel are transferred on a nitrocellulose membrane. Take a wick that touches the buffer and put it on the gel. The buffer is 80% SDS running solution and 20% methanol. Then take a nitrocellulose membrane and lay it over the wick and the gel. Apply the western blotting machine for five minutes to transfer the proteins from the gel to the membrane. After one transfer incubate overnight in 3%BSA/TBS overnight at 4°C on an orbital shaker at 50 rpm. Wash in readiness for primary antibody detection with biotin conjugate (TG 100). Add 3 µl of the primary antibody and incubate for two hours at room temperature. Wash the membrane in an orbital shaker at 50 rpm to ensure even washing, for ten minutes six times in 20 ml of TBS or PBS Tween.

The membrane is now ready for secondary antibody detection anti-mouse anti IgG (whole molecule) Peroxidase antibody which produced in goat (Sigma Aldrich, St. Louis, MO, United States of America. Dilute the antibody with BSA/TBS at a rate of 1 in 2000 in 3% BSA/TBS solution.). After that add 3 µl of the secondary antibody and then incubate it at room temperature for three hours. Wash the membrane again six times for ten minutes in 20 ml of TBS or PBS Tween to remove any unbound secondary antibody. Remove the excess buffer from the membrane and detect the TG2 by use of ECL which obtained via Amersham ECL Prime Western Blotting detection Reagents kit (Bio-Rad Laboratories, Inc., Hercules, CA, United States of America). Read the result by using BIO-RAD visualizer.

2.6 Non Denaturing Gel Page:

In non-denaturing poly acrylamide gel electrophoresis, a denaturing enzyme is omitted in the procedure. The method uses two gels that is the resolving gel and the stacking gel to do the separation. The presence of chloride ions modifies the pH making the glycine to be ionized. This creates a voltage gradient which allows migration of proteins hence separation by size.

2.6.1 Materials

Laboratory Equipment
Glass plates(a small and a large plate)
Casting frame
Casting stand
Combs

Non-denaturing PAGE Equipment

Reagents
1.5 M Tris, pH 8.9
0.5 M Tris, pH 6.8
Isopropanol / distilled water
Running buffer
Coomassie Brilliant Blue dye
Protein samples
30 % polyacrylamide solutions
TEMED
10% ammonium persulfate

Reagent for SDS-PAGE

2.6.2 Method

Running Buffer:

A running buffer should have a PH of 8.3, that is (10x stock) 6.0g Tris, 28.4g glycine dissolved in 1.0 litres. To prepare smaller volume of the buffer for instance 100ml (10x): pH 8.3 dissolve 0.6g Tris and 2.84g glycine in 100ml.

(A) Resolving Buffer 1.5M Tris/HCl pH 8.9

(B) Stacking Buffer 0.5M Tris/HCl pH 6.7

(C) Acrylamide

Resolving Mixture	**7.5%**	**Stacking gel**	
(A)	5.0	(B)	5.0
(C)	5.0	(C)	2.5
H_2O	9.9	H_2O	12.4
+10% (w/v) AmmperSulph (0.1g in 1.0ml)	0.06	+10% (w/v)Amm persulphate	0.06
TEMED	0.04	TEMED	0.04
Total	20.0ml	**Total**	20.0ml

Sample Buffer:

To prepare 5.0ml (stacking buffer) add the following reagents;

2.0ml glycerol + 1.0ml 2-mercaptoethanol (2-ME) + 11ml H_2O and the Put samples on ice before loading onto the gel.

Prepare the Resolving gel and pour it 1 cm below the comb. Overlay the gel with isopropanol and allow the gel to polymerise. After polymerization, discard the solvent and wash the gel with distilled water. Using the blotting paper to remove the water and avoid touching the gel following insert the Teflon well forming the comb. After the resolving gel has formed, prepare the stacking gel. Add the catalyst to the stacking gel and pour onto the resolving gel until it reaches the top of the plate. Overlay it with isopropanol and allow it to polymerise. Upon polymerization, remove the comb and wash with distilled water to remove unpolymerized acrylamide. Both the stacking and the resolving gel are ready for loading with samples. Fill the

wells with the running buffer and load the samples. One well should be loaded with dye to help determine the running time. Run the gel at 40 ma per gel for one hour. Stop the reaction when the bromophenol blue reaches 0.5 cm from the end of the gel. Remove the gel from the tray and cover it with safe blue stain for two hours. Remove the gel from the stain and place it in destain until the background is clear enough to visualise the samples.

2.7 Transglutaminase 2 Assay (Biotin Cadaverine)

This assay was used to determine the activity of transglutaminase 2 and it has been performed according to slaughter et al (1989).

2.7.1 Material

Laboratory Equipment	**Reagents**
96 well plate	Tris/Hcl pH 8.5
Microfuge tubes	NN-dimethyl casein
250 µl pipette	BSA 3%
10 µl pipette	2-marcaptoethanol
1000 µl pipette	Biotin cadaverine
Incubator	Extravedine peroxidase
Distilled water	Calcium tris buffer pH 8.5
Stop watch	EDTA tris buffer pH 8.5
Red tubes	Sodium acetate buffer pH 6.0
Tissue paper	TMB
Racks	Sulphuric acid

	TG2 gunie pig liver
	DMSO
	Different concentrations of methanol
	Fractions peptides

TG2 assay equipment *TG2 assay reagents*

2.7.2 Method

Pipette 250 µl of 10 ml 1-NNdimethyl casein into each well of a 96 well plate and incubate overnight at 4°C. After overnight incubation remove the reagent and wash with distilled water after that discard the water into the sink. Repeat that three times and allow the plate to dry and then block the plate by adding 250 µl of 0.1 M Tris/HCl pH 8.5 with 3% (w/v) BSA to all wells and leave it for 45 minutes at room temperature. Wash the plate with distilled water and discard the water then leave it to dry on tissue paper. Repeat the washing three time. Later take 10 ml of calcium Tris buffer and add 10 µl of 2-mercaptoethanol and 10 µl of biotin cadaverine. Then take 10 µl of EDTA Tris buffer and add 10 µl of 2-mercaptoethanol and 10 µl of biotin cadaverine. Keep the two buffers away from each other. Take the plate and add 150 µl of calcium buffer to the top four rows and an equal amount of EDTA buffer in the bottom four rows. Prepare the dilutions of TG2 and keep it on the ice.

2.7.3 Sample preparation of TG2:

2.7.4 Casein peptides samples

Take seven microfuge tubes and label them from A – G, Afterward take 25 µl of gpl and add this to 25 µl of 0.1 M Tris buffer pH 8.5 into (A). Then add 25 µl of gpl and 25 µl of unbound

material to (B). In (C) add 25 µl of gpl and 25 µl of 10% methanol, Add 25 µl of gpl and 25 µl of 25% to (D), and then in (E) add 25 µl of gpl and 25 µl of 50% of methanol. Afterward add to (F) 25 µl of gpl and 25 µl of 80% of methanol. At the last microfuge tube (G) add 25 µl of gpl and 25 µl of 100% of methanol, after that place all the samples in ice.

2.7.5 Sample preparation of DMSO:

Firstly, label 5 microfuge tubes from A-E, then add 25 µl of gpl and 25 µl of DMSO 0.0125 µM [I_1] to microfuge tube (A), after that add 25 µl of gpl and 25 µl of DMSO 0. 125 µM [I_2] to (B), In microfuge tube (C) add 25 µl of gpl and 25 µl of DMSO 1.25 µM [I_3], and add 25 µl of gpl and 25 µl of DMSO 12.5 µM [I_4] to (D). Finally add 25 µl of gpl and 25 µl of DMSO 125 µM [I_5] to (E). Then place all the samples in ice.

2.7.6 Add samples to the plate:

At the 1st column add 50 µl of 0.1 Tris buffer pH 8.5, and in the 2nd column add 50 µl of A (gpl samples as it shows in method 2.7.4), in the 3rd column add 50 µl of B, and also add 50 µl of C in the 4th column, then add 50 µl of D in the 5th column, in the 6th column add 50 µl of E. and 50 µl of F to the column 7. Lastly, add 50 µl of G to the column 8.

Repeat the same steps to add DMSO samples into the plate.

Cover the plate and incubate for 37°C for 60 minutes. Prepare extravidin peroxidise solution by adding 25 ml of 0.1M Tris buffer at pH 8.5 and also add 5 µl of extravidin peroxidise. After 60 minutes of incubation discard the buffer and wash it as before allowing it to dry. Add 200 µl of the extravidin solution to each well and incubate at room temperature for 45 minutes. Then wash the plate as before and allow it to dry. Then start preparing the development assay in 50 ml red

tube by adding 25 ml of 0.1 M of sodium acetate buffer pH 6.0, add 3 µl of 30% of (v/v) H_2O_2, then add 150 µl of TMB. In every 10 seconds start adding 200 µl of development buffer to the first well then move to the next well until the first three columns completed. Using a stopwatch time the reaction at ten-second interval for the reaction by adding 50ul of 5M sulphuric acid. Repeat to ensure all wells have incubated. After incubation the plate is ready to be read at a wavelength of 450nm.

2.8 Deamidation Assays

2.8.1 Ammonia Standard Assay:

2.8.2 Material

Laboratory Equipments	**Reagent**
50 mL red tube	OPA
200 multi-channel pipette	Sodium borate
1000 µl pipette	Ultra-pure water
96 well black micro plate	Sodium sulphite
A volumetric flask	ammonium chloride
25 µl pipette	
Micro plate fluorescence reader	

Standard ammonium equippemnts *Reagents for standard ammonium*

2.8.3 Method

Standard working reagent can be prepared by adding 500 ml of sodium borate, 2.5 ml of sodium sulphite and 25 ml of OPA to the storage bottle and then cover the bottle with aluminium foil to preserve the solution from the light. Measure 100 mM of ammonium chloride and dissolve it in ultra-pure water. Take a volumetric flask and wash it with 0.1 M (HCL), then leave it to dry on tissue paper. Fill the volumetric flask with 250 ml of ammonium chloride and after that transfer

the solution to the storage bottle. From this concentration prepare different concentration of ammonium chloride (1mM, 0.1mM, 0.01mM, and 0.001mM). To prepare theses concentrations, firstly, add 0.1 ml of ammonium chloride solution to 9.9 ml of ultra-pure water to make 1 mM (A), then take 0.2 ml of (A) and add it to the 1.8 ml of ultra-pure water to prepare 0.1 mM (B). From (B) take 0.2 ml and add it to 1.8 ml of ultra-pure water for 0.01 mM concentration (C). From (C) take 0.2 ml and add it to 1.8 ml of ultra-pure water to make 0.001 mM (D). Divide the plate into two parts, the upper part is from (A-D) and the lower part is from (E-H). In the upper part fill it with 1 mM of ammonium concentration and amount of ultra-pure water to the first six columns. Afterward add 0.1 mM of ammonium concentration to the lower part of the plate and add to this ultra-pure water. Repeat these steps but now with different concentrations of ammonium chloride (0.01 mM and 0.001 mM). Then add 25µL of each dilution into black fluorescence 96 well plates supplemented with 200 µL of standard working reagent. Incubate each dilution of ammonium chloride at 60 °C for 20 minutes, and then release ammonia in fluo star optima 96 well plate reader relative fluorescence unit at lamda ex=360nm and lamda em =430nm.

2.8.4 Fluorescence Plate Assay

2.8.5 Material

Laboratory Equipment	**REAGENT**
96 well black micro	Distilled water

plate	
Centrifuge tubes	Calcium buffer
Micro pipette	EDTA buffer
15mL Red bottles	Storage protein
Incubator	NN-dimethyl casein
	BSA 3%
	Polyamines (Putrecine, Sperimine, Sperimidine, Cadaverine, Methylamine

Fluorescence assay equipment *Fluorescence assay reagents*

2.8.6 Method

First and foremost, prepare 10 ml of 10 mM of putrecine, 10 mM of sperimine, 10 mM of sperimidine, 10 mM of cadavarine and 10 mM of methylamine in 15 ml of red tubes. From these solutions, prepare 1 mM stock of all five polyamines in microfuge tubes and label them from (1-5) respectively by adding 100 µl of polyamines to 900 µl of ultra-pure water. Coat the clear plastic plate with 250 µl of 10 ml 1-NNdimethyl casein and leave it overnight at 4°C, later, wash it three times then add 250 µl of 0.1 M Tris/HCl pH 8.5 with 3% (w/v) BSA. Leave it at room temperature for 45 minutes; afterward wash it as before three times.

Start adding 150 µl of calcium buffer with 10 µl of 2-mercaptoethanol and 10 µl of biotin cadaverine to the first four rows and 150 µl of EDTA buffer with 10 µl of 2-mercaptoethanol and 10 µl of biotin cadaverine to the second four rows. Add at the first column water. Next, add 20 µl of plyamines (putrecine, sperimine, sperimidine, cadavarine and methylamine) to the columns

from 2-6 respectively. Then add 25 µl of TG2 and 5 µl of water to all wells. Put it in the incubation at 37°C for an hour. After the incubation put the plate in the ice, and transfer 25 µl of sample by using multichannel Pipette to the black plate then add to this 25 µl of water and 200 µl of standard working reagent. Incubate the plate at 60 °C for 20 minutes. After incubation the plate is ready to be read by omega fluorescence reader.

2.9 Measure the mobility in non-denaturing PAGE gel:

2.9.1 Sample Preparation of Different Types of TG2

Prepare 10 glass vails and label them from 1-10, put them in rack, then make up calcium buffer and EDTA buffer with 10 µl of 2-mercaptoethanol. In the 1st glass vail add 200 µl of calcium buffer, 50 µl of storage protein and 25 µl of water (control). In 2nd glass vail add 200 µl of calcium buffer, 50 µl of storage protein and 25 µl of sigma TG2. Add 200 µl of EDTA buffer, 50 µl of storage protein and 25 µl of sigma TG2 to the 3rd glass vail. Then add 200 µl of calcium buffer, 50 µl of storage protein and 25 µl of zedira TG2 to the 4th glass vail. In the 5th glass vail add 200 µl of EDTA buffer, 50 µl of storage protein and 25 µl of zedira TG2. Add to the 6th glass vail 200 µl of calcium buffer, 50 µl of storage protein and 25 µl of pure TG2. In the 7th glass vail add 200 µl of EDTA buffer, 50 µl of storage protein and 25 µl of pure TG2. While adding 200 µl of calcium buffer, 50 µl of storage protein and 25 µl of gpl TG2 in the 8th glass vail, add 200 µl of EDTA buffer, 50 µl of storage protein and 25 µl of gpl of TG2 to the 9 th glass vail. And in the last glass vail add 200 µl of EDTA buffer, 50 µl of storage protein and 25 µl of water (control). Incubate them at 37°C overnight.

2.9.2 Sample preparation of TG2 inhibitors:

Take 8 small glass vails and put them in the rack then label them. In all of them add 200 µl of calcium, 50 µl of storage protein and 25 µl of gpl. However, in the 1st glass vail add 25 µl of cystamine, in the 2nd glass vail add 25 µl of Z_DON while in the 3rd glass vail add 25 µl of R 283. Add 25 µl of polyamines (putrecine, sperimine, sperimidine and cadavarine) to the 4th, 5th, 6th, 7th glass vails respectively. Afterward add 25 µl of EDTA to the 8th glass vail and add 25 µl of water to the last glass vail. Next, 37°C overnight incubation is needed.

2.9.3 Sample preparation of different types of proteins:

To the 1st glass vail add 25 µl of storage protein and 100 µl of calcium Tris buffer. In the 2nd glass vail add 25 µl of storage protein and 100 µl of EDTA buffer. Add 25 µl of α crystalline and 100 µl of calcium buffer to the 3rd glass vail. Then in the 4th glass vail add 25 µl of α-crystalline and 100 µl of EDTA buffer. Afterward, Add 25 µl of β-crystalline and 100 µl of calcium buffer to the 5th glass vail, then add Add 25 µl of β -crystalline and 100 µl of EDTA buffer to the 6th glass vail. In the 7th glass vail add Add 25 µl of elafin and 100 µl of calcium buffer. Lastly, add 25 µl of elafin and 100 µl of EDTA buffer to the 8th glass vail. Then add to all these glass vails 12.5 µl of human recombinant and 12.5 µl of water. Next, incubate it at 37°C overnight.

2.9.4 Sample preparation of Elafin inhibitors

Take 100 µl of calcium buffer and add to this 25 µl of storage protein, 12.5 µl of human recombinant TG2 and 12.5 µl of elafin to the 1st glass vail. Next, add 100 µl of calcium buffer, 25 µl of storage protein, 25 µl of water to the 2nd glass vail. In the 3rd glass vail add 100 µl of EDTA buffer and add to this 25 µl of storage protein, 12.5 µl of human recombinant TG2 and 12.5 µl of

elafin. In the last glass vail add 100 µl of EDTA buffer, 25 µl of storage protein, 25 µl of water. Incubate them at 37°C overnight.

2.9.5 Loading the samples:

After overnight incubation, take 40 µl of samples which are prepared above and add it to this 10 µl of bromophenol blue stain. Then put in ice, and start loading the samples by adding 10 µl of samples to each well. Run the gel at 40 mAmps for each gel for an hour. Finally, the gels are ready for reading by using Bio-Red visualizer.

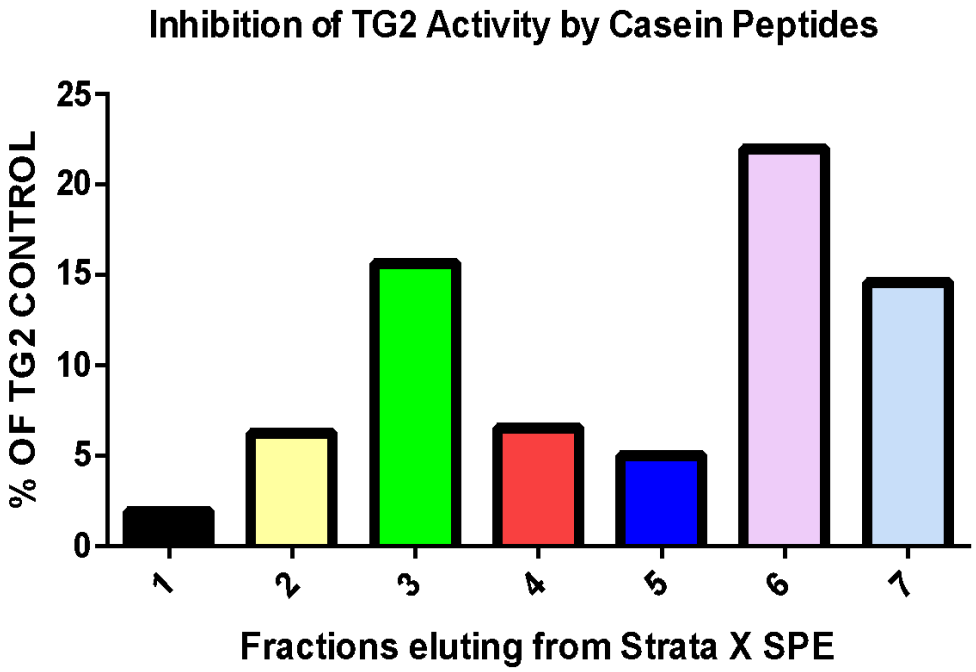
CHAPTER: 3

RESULTS:

3.1 Transglutaminase2 Assay:

The GraphPad prism was used to analyze the data in Transglutaminase2, Ammonia and Fluorescence assays. Transglutaminase2 assay was utilized to measure the activity of transglutaminase2. Figure 1 below shows the highest percentage of TG2 control occurred in fraction 6 which represented 80% of methanol and followed by fraction 7 that represented 100% of methanol; this is an indication that increase in methanol concentration induced higher TG2 activity. Therefore, the inhibition of TG2 is not occurred. On the other hand, inhibition was high in fraction 5 (50% methanol) followed by fraction 4 (25% methanol). It is important to note that at 50% of concentration of methanol is relatively affected the TG2 activity.

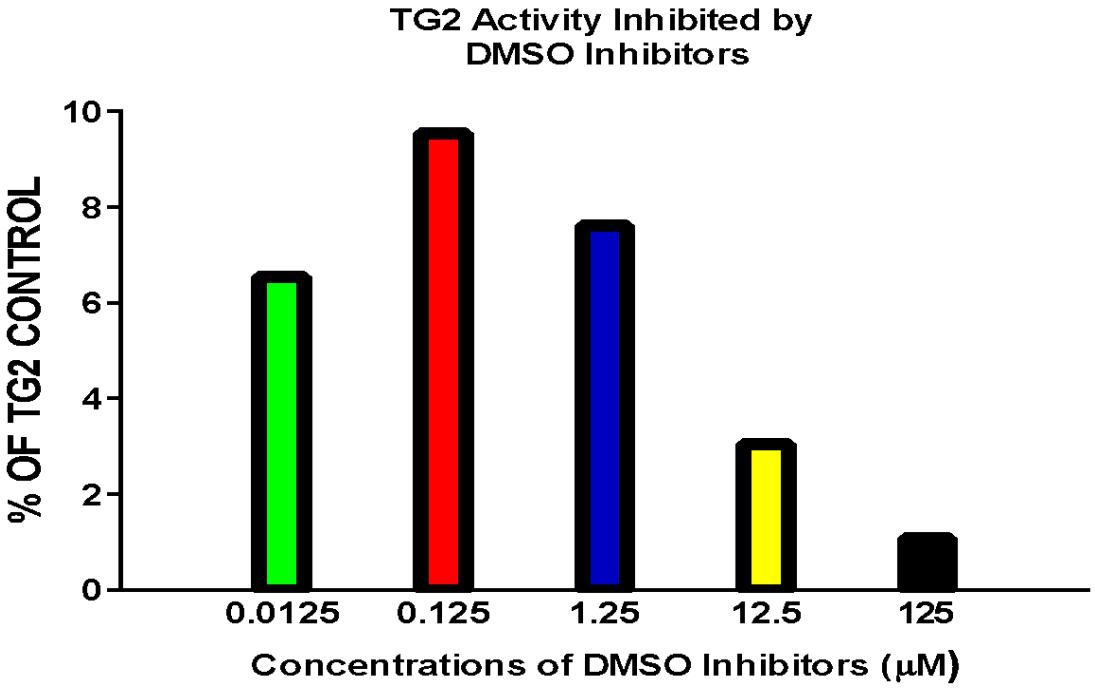
Figure.1- Shows that TG2 activity is inhibited by casein peptides



Transglutaminase2 assay was performed according to method and material 2.7.4. Casein peptides with different concentrations of methanol were added into the wells of the plate. Fraction 1= Buffer. Fraction 2= unbound material. Fraction 3= 10% of methanol. Fraction 4= 25% of methanol. Fraction 5= 50% of methanol. Fraction 6= 80% of methanol. Fraction 7= 100% of methanol. Gpl was added to all wells. The plate was incubated at 37◦C for 60 minutes and then the development buffer was added to the wells to start the reaction and stopped by sulphuric acid. The plate was read by using micro plate reader.

The finding in figure 2 indicates that DMSO inhibited the control level of TG2. It is evident that the increase in concentration of DMSO reduced the percentage of TG2 control. For instance, the least inhibition occurred in DMSO of concentration 0.125 µM whereas the highest inhibition occurred in DMSO of concentration 125 µM. It is important to note that the relationship between the concentrations of DMSO and percentage of TG2 control is near to be linear. However, there was no inhibition occurred in the other concentrations while the highest concentration of DMSO induced relatively inhibition of TG2 activity.

Figure.2- Inhibition of TG2 activity by different concentrations of DMSO inhibitors

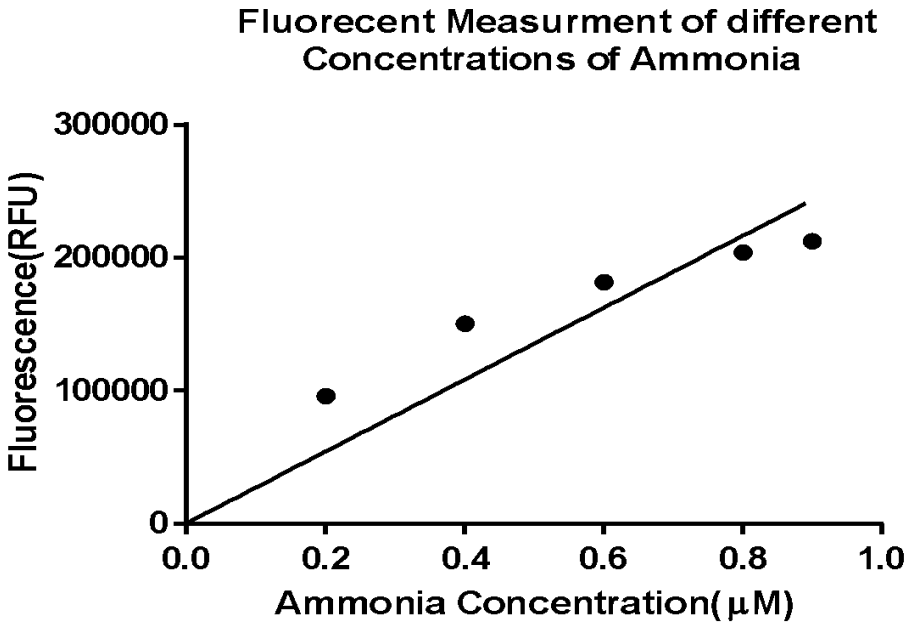


Transglutaminase2 assay was done according to method and material 2.7.5. Different concentrations of DMSO were added into the wells of the plate. DMSO inhibitor1= 0.0125 µM. DMSO inhibitor2= 0.125. DMSO inhibitor3= 1.25 µM. DMSO inhibitor4= 12.5 µM. DMSO inhibitor5= 125 µM. Gpl was added to all wells. The plate was incubated at 37◦C for 60 minutes and then the development buffer was added to the wells to start the reaction and stopped by sulphuric acid. The plate was read by using micro plate reader.

3.2 Standard Ammonium Assay

Transglutaminase2 assay has been given negative result. Therefore, ammonia assay was performed. The fluorescent reaction was occurred through the reaction of ammonia released and orthophthalaldehyde (OPA). It is evident from the two graphs that the relationship is linear since nearly all the plotted points fall near the linear trendline and thus indicates that the RFU measurement of ammonia is moderately proportional to its concentration. Since most of the plots are close the linear trendline (Figure 3 and Figure 6).

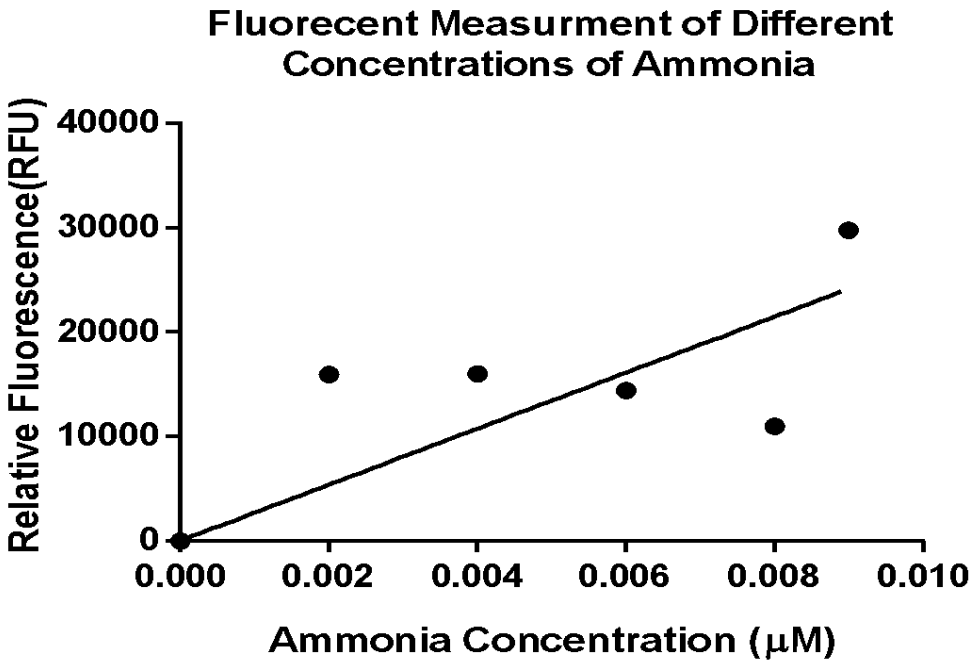
Figure.3- Shows the linear relationship between RFU and different concentrations of ammonia chloride at (0.001mM)



Ammonia assay was performed according to method and material 2.8.3. Ammonia chloride at concentration 0.001 mM involved adding 25 µL of diluted ammonium chloride and 200 µL of standard working reagent into fluorescence 96-well fluorescent plate. The plate was incubated at 60◦ C for 20 minutes. Fluorescence reading was done in fluo star optima 96-well plate reader with RFU at lamda ex=360nm and lamda em =430nm.

However, the graph for figure 4 indicates that the relationship is non-linear because no plotted points appear near the linear trend line and thus indicates that the RFU measurement of ammonia is not directly proportion to its concentration. Therefore, it exhibits weaker linear correlation.

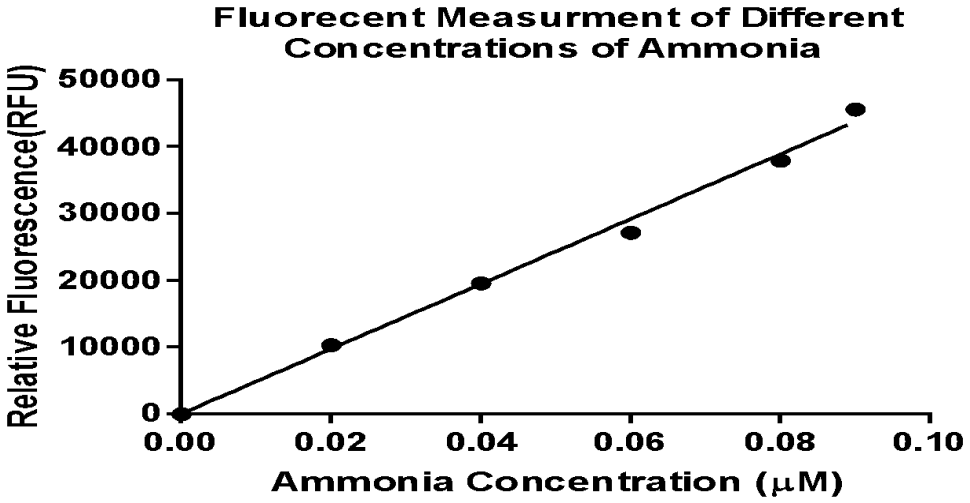
Figure.4- Demonstrates the linear relationship between RFU and different concentrations of ammonia chloride at (0.01mM)



Ammonia assay was done according to method and material 2.8.3. Ammonia chloride at 0.01 mM involved adding 25 µL of diluted ammonium chloride and 200 µL of standard working reagent into fluorescence 96-well fluorescent plate. The plate was incubated at 60◦ C for 20 minutes. Fluorescence reading was done in fluo star optima 96-well plate reader with RFU at lamda ex=360nm and lamda em =430nm.

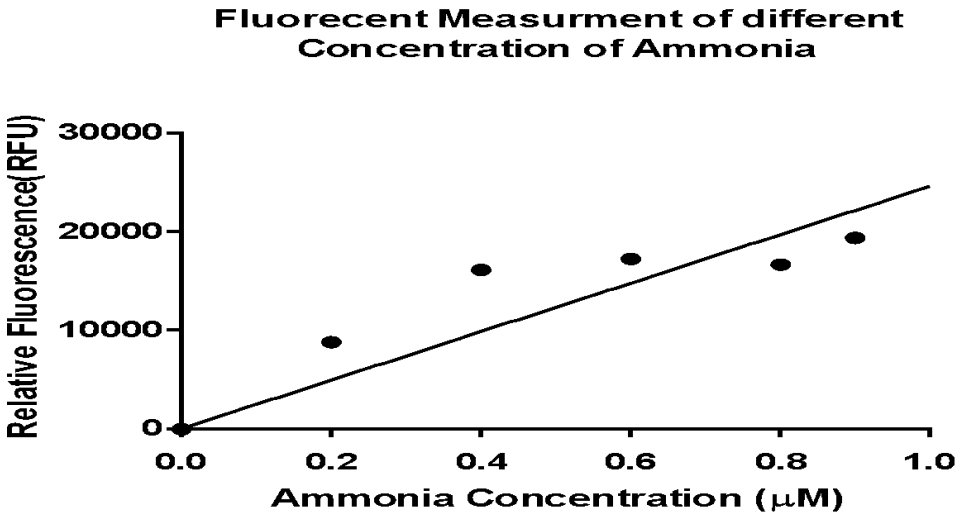
In addition, in figure 5 it is evident from the graph that this relationship is linear since all the plotted points fall within the linear trend line and thus indicates that the RFU measurement of ammonia is directly proportion to its concentration. Since all of the plots are within the linear trend line, it indicates strong correlation between RFU and ammonia concentration.

Figure.5- Explains the linear relationship between RFU and different concentrations of ammonia chloride at (0.1mM)



Ammonia assay was performed according to method and material 2.8.3. Ammonia chloride at concentration 0.1 mM involved adding 25 µL of diluted ammonium chloride and 200 µL of standard working reagent into fluorescence 96-well fluorescent plate. The plate was incubated at 60◦ C for 20 minutes. Fluorescence reading was done in fluo star optima 96-well plate reader with RFU at lamda ex=360nm and lamda em =430nm.

Figure.6- Shows the linear relationship between RFU and different concentrations of ammonia chloride at (1mM)

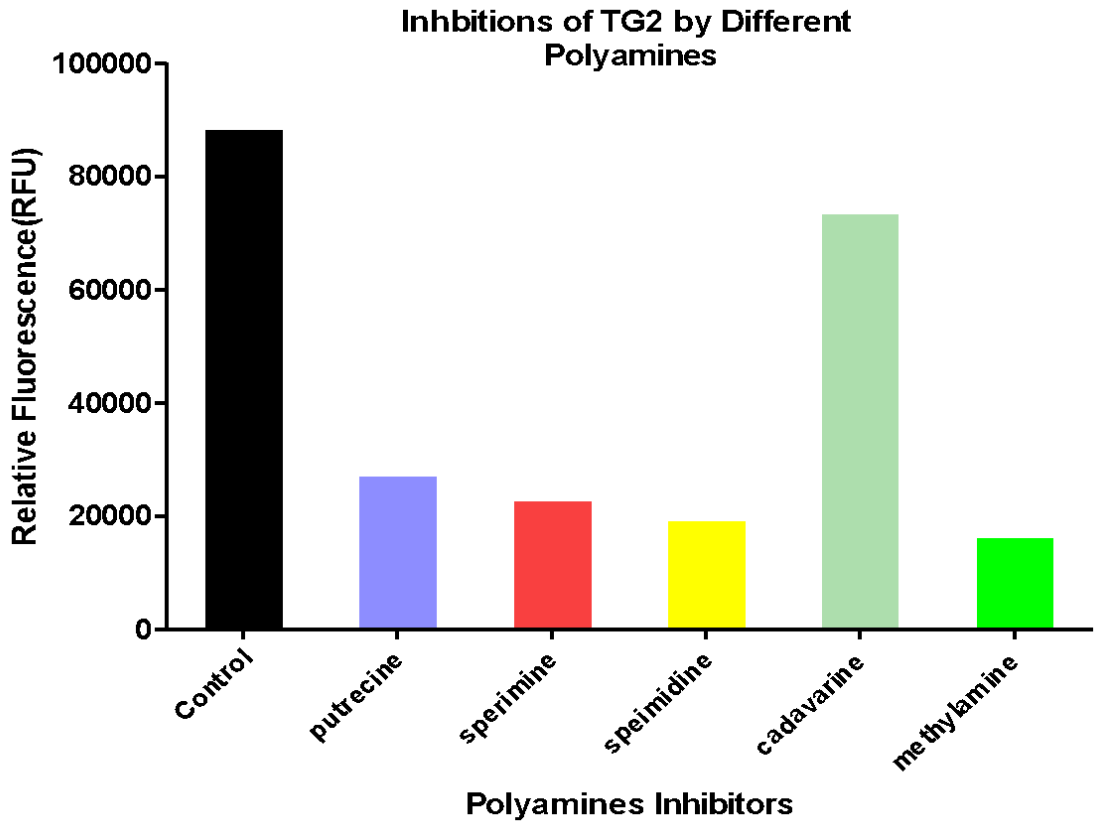


Ammonia assay was done according to method and material 2.8.3. Ammonia chloride at concentration 1 mM involved adding 25 µL of diluted ammonium chloride and 200 µL of standard working reagent into fluorescence 96-well fluorescent plate. The plate was incubated at 60◦ C for 20 minutes. Fluorescence reading was done in fluo star optima 96-well plate reader with RFU at lamda ex=360nm and lamda em =430nm.

3.3 Fluorescence Assay:

Fluorescence assay was done to assess the activity of TG2. Polyamines are organic compounds with two or more primary amino (NH_2) groups (Ohtake et al. 2007). The finding in Figure 7 presents inhibition of TG2 by different polyamines. It is evident that the activity of TG2 seems to be negatively inhibited by polyamines – putrecine, spermidine, spermine and cadaverine whereas the most inhibition activity had occurred to methylamine. It is also important to note that the control was higher than polyamines.

Figure.7- The inhibition of TG2 activity by using different polyamines

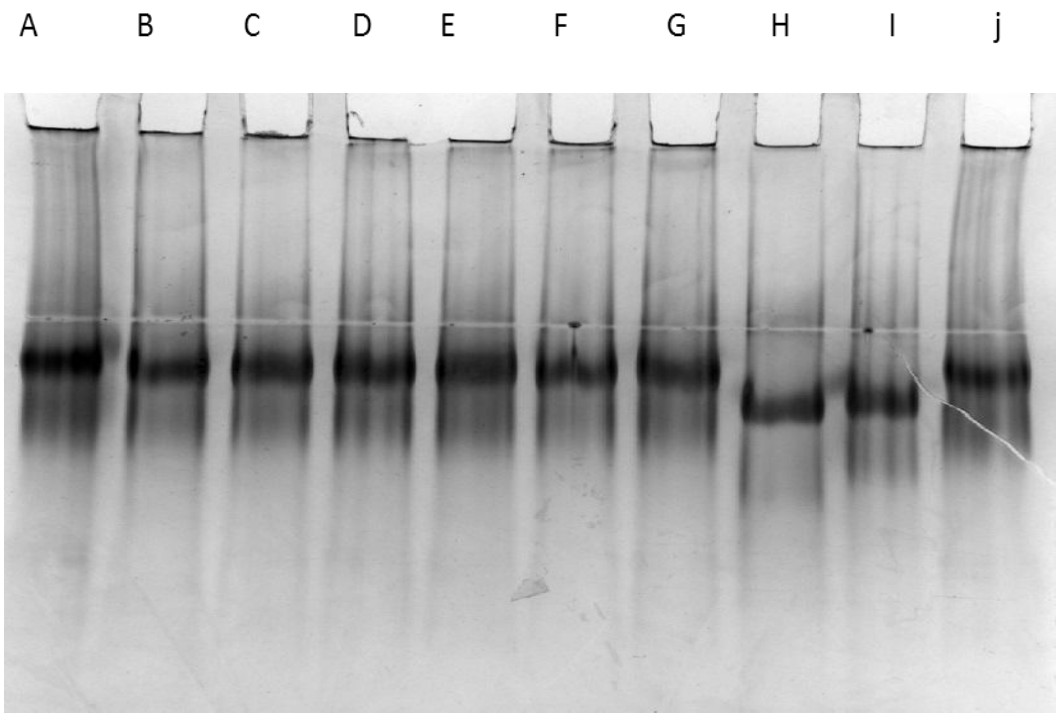


Fluorescence assay was performed according to method and material 2.8.6. Different polyamines were added into the wells of fluorescent plate. 1= water. 2= Putrecine. 3= Sperimine. 4= Sperimidine. 5= Cadaverine. 6= Methylamine. Then TG2 were added to all wells and after incubation at 37°C for an hour, the sample was transferred to the fluorescent plate. Then the plate was incubated at 60 °C for 20 minutes. The plate was read by omega fluorescence reader.

3.4 Non-denaturing gel PAGE:

Fluorescence assay was done and it has not shown a positive result and thus the Non-denaturing gel electrophoresis was performed. Figure 8 shows that storage protein with calcium, EDTA and gpl were moved further in the gel and thus indicate that the increasing the deamidation of protein. On the other hand, the deamidation distance was equal on the other wells. Therefore, no deamidation occurred in the other wells.

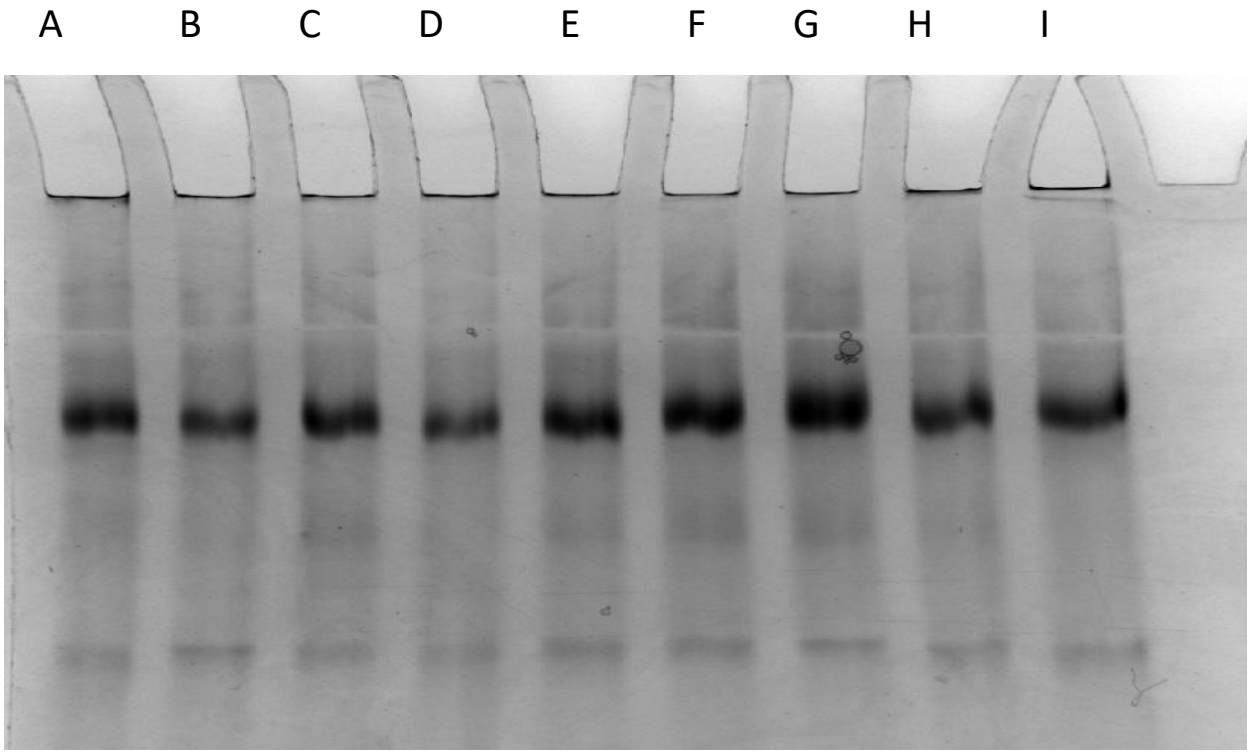
Figure.8- Non-denaturing gel electrophoresis to assess the deamidation of different proteins with calcium and EDTA



Non-denaturing gel electrophoresis was performed according to method and material 2.9.1. The arrangement of the samples in the wells included the following based on labels (A) Control , (B) ca^{+2}+Sigma, (C) EDTA+Sigma, (D) ca^{+2}+TG2, (E) EDTA+TG2, (F) ca^{+2}+Pure TG2, (G) EDTA+Pure TG2, (H) ca^{+2}+gpl, (I) EDTA+gpl, (J) Control. The gel was run at 40 ma per gel for 60 minutes and stained in safe blue stain for an hour. Then the photograph was taken by using Bio-Rad visualizer.

In figure 9 this was done in the presence of the inhibitors such as cystamine, Z-DON, R283, putrecine, sperimine, sperimidine and cadaverine, these inhibitors are prevented the deamidation of the storage protein. Therefore, the deamidation movement did not occur in any of the wells.

Figure.9- Non-denaturing gel electrophoresis to assess the deamidation of proteins by using different inhibitors

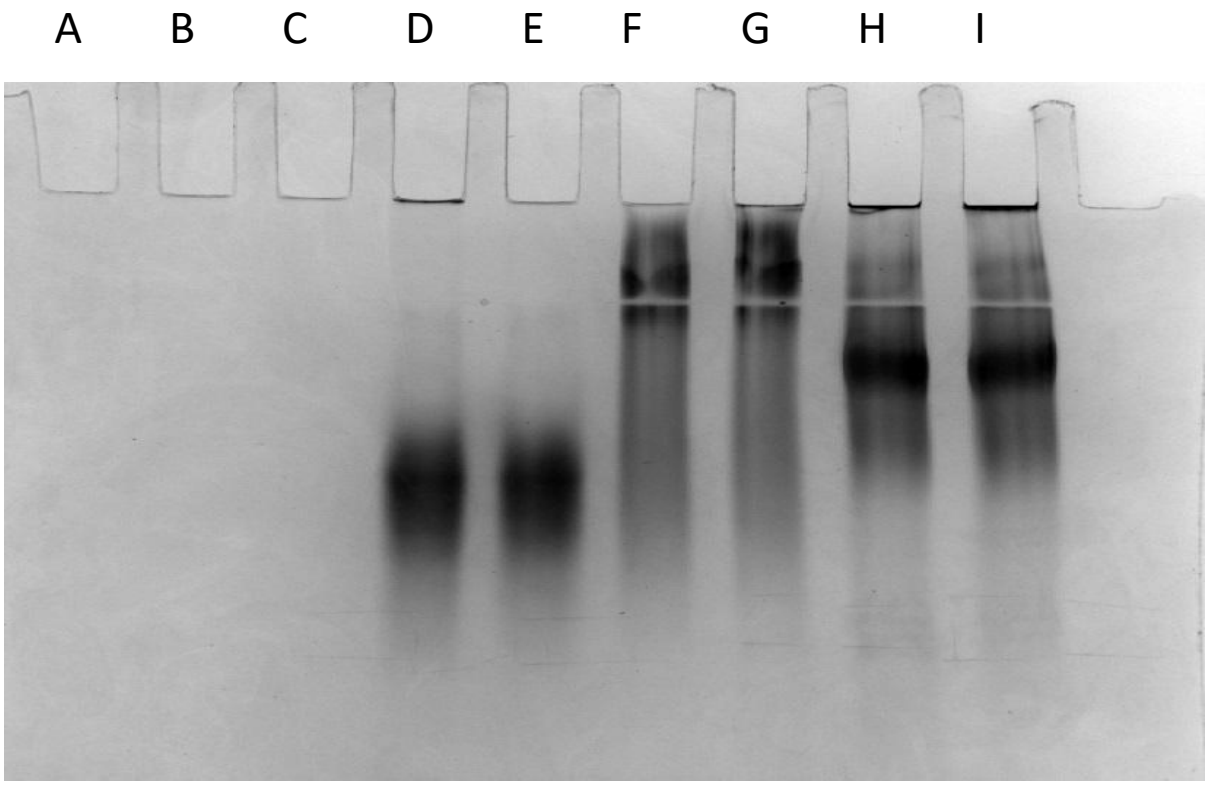


Non-denaturing gel electrophoresis was done according to method and material 2.9.2. The arrangement of the samples in the wells included the following based on labels. (A) Cystamine, (B) Z-DON, (C) R283, (D) Putrecine, (E) Sperimine, (F) Sperimidine, (G) cadaverine, (H) EDTA, and (I) water. The gel was run at 40 ma per gel for 60 minutes and stained in safe blue stain for an hour. The photograph was taken by using Bio-Rad visualizer.

The figure 10 below shows that the deamidation of protein was found in EDTA and calcium β-crystaline with TG2 human recombinant which is moved further in the gel. This migration strongly indicates that protein structure affected the nature of deamidation. In contrast, storage

protein and α-crystaline did not show any deamidation. Furthermore, the Ca^{+2}+Elafin and EDTA+Elafin were not appeared in the gel.

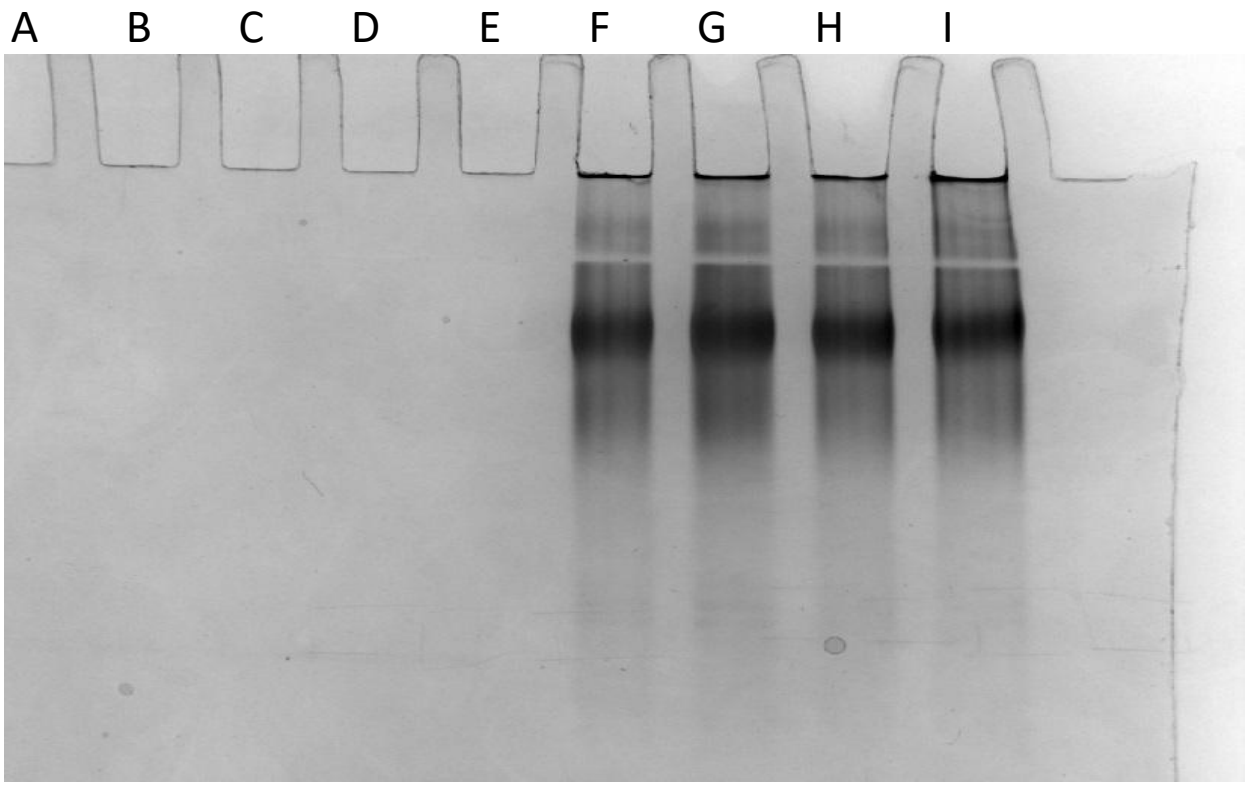
Figure.10- Non-denaturing gel electrophoresis to assess the deamidation of different proteins with calcium and EDTA



Non-denaturing gel electrophoresis was done according to method and material 2.9.3. The arrangement of the samples in the wells included the following based on labels. (B) Ca^{+2}+Elafin, (C) EDTA+Elafin, (D) Ca^{+2}+ β-crystaline, (E) EDTA+β-crystaline, (F) Ca^{+2}+ αcrystaline, (G) EDTA+α-crystaline, (H) Ca^{+2}+ storage protein, and (I) EDTA+storage protein. The gel was run at 40 ma per gel for 60 minutes and stained in safe blue stain for 60 minutes. The photograph was taken by using Bio-Rad visualizer.

Figure 11 demonstrates that this is done in the presence of elafin inhibitor in the wells (G and I). All the wells exhibited the same migration distance and thus indicate no deamidation occurred in all of the wells. In addition, in the wells calcium and EDTA elafin protein were not exhibited in the gel. This suggests that the control had the same effects either in the presence or absence of elafin inhibitor.

Figure.11- Non-denaturing gel electrophoresis to assess the deamidation of proteins as influenced by alafin inhibitors with calcium and EDTA



Non-denaturing gel electrophoresis was performed according to method and material 2.9.4. The arrangement of the samples in the wells included the following based on labels. (D) EDTA+Elafin protein, (E) Ca^{+2}+ Elafin protein, (F) EDTA Control, (G) EDTA+{I} elafin, (H) Ca^{+2} Control. (I) Ca^{+2}+{I} Elafin. The gel was run at 40 ma per gel for 60 minutes and stained in safe blue stain for 60 minutes. The photograph was taken by using Bio-Rad visualizer.

DISCUSSION

Transglutaminase 2 (TG2) is a ubiquitous enzyme that performs a range of functions in almost every part of the body (Hasegawa et al, 2003). Its main function is to cross-link glutamine residues on various proteins and bring them together to form a complex aggregate responsible for important cellular functions. In addition, it can also function as a G protein and protein kinase (Mishra and Murphy, 2004). High levels of TG2 are implicated in several diseases such as Huntington's disease, bronchopulmonary dysplasia, and celiac disease. Hence, the inhibition of TG2 in these diseases is an actively explored option for possible therapeutic breakthroughs (McConoughey et al, 2010).

The activity of TG2 can be deregulated by several inhibitors and these include R2 peptides, cystamine (CTM) and dexamethasone (Sohn et al, 2010). Given that TG2 has important roles in normal physiological processes of the body and in several disease states, the search for more potent and stable inhibitors of TG2 is carried out with the objective of identifying attractive therapeutic options (McConoughey et al, 2010). At the same time, it is also important to be able to measure inhibition caused by novel inhibitors on transglutaminase activity.

In the current work undertaken, the enzyme TG2 was first partially purified from guinea pig liver. The TG2 assay was carried out in an attempt to test for the inhibition of the enzyme by casein peptides and DMSO (Liu et al, 2002). This assay aimed to identify the activity of the enzyme by mixing casein peptides with different concentrations of methanol and measuring the activity of the enzyme. If inhibition had occurred, there would be no activity or very little activity of the enzyme and vice versa (Wong et al, 2002).

From the results obtained, a lower concentration of casein peptides (0 – 20%) produced no inhibition of TG2 as indicated by a considerable amount of TG2 (15 – 25%) measured in the assay. On the contrary, a higher concentration of casein peptides (50 – 75%) produced considerable inhibition of the enzyme as only 5 – 10% of the enzyme was measured. Hence, fraction 3 used in the experiment was able to provide inhibition of transglutaminase activity at 50% of casein peptide concentration. This assay gave a linear regression response as decrease in the concentration of casein peptides resulted in increasing concentrations of TG2 and complete inhibition of transglutaminase activity occurred only at 50% of casein peptides. This was expected as casein has long been used in transglutaminase assays as an inhibitor of the activity of TG2 at standardized concentrations (Liu et al, 2002).

The same result was obtained when varying concentrations of DMSO were used to test the concentration of DMSO where inhibition occurred. From the results obtained, the lowest concentration of TG2 was observed at the highest concentration of DMSO used (125 μM) and the highest concentration of TG2 was observed at 0.125 μM of DMSO. Hence, a concentration of 125 μM of DMSO caused the greatest inhibition of transglutaminase activity. The result of this assay also follows the linear regression model where inhibition of transglutaminase activity occurred at 125 μM of DMSO. Hence, the TG2 assay to test the activity of TG2 in the presence of casein peptides or DMSO clearly indicate that both casein peptides and DMSO can lead to inhibition of TG2 activity at 50% and 125 μM respectively. This is in accordance with literature which suggests that DMSO in the presence of $CaCl_2$ can significantly inhibit the activity of transglutaminase (Siegel et al, 2008). In case of certain neuronal cell lines, DMSO alone was also able to produce inhibition of transglutaminase activity (Milakovic et al, 2004).

However, there were certain inconsistencies with the TG2 assay. In case of casein peptides, there was a sudden increase in the concentration of TG2 at 90% casein peptide concentration, which does not follow the rules of a linear regression model. Also, in case of DMSO, the inhibition of TG2 was higher at 0.0125 μM than 0.125 μM, which is again contrary to the linear regression model. Hence, the ammonia assay, which is a modification of the transglutaminase 2 assay, was performed to assess the inhibition of TG2. Here, ammonium ions are released upon deamidation of the substrate by transglutaminase (Schmid et al, 2011). These ions released in the reaction would serve to stop the reaction and the fluorescence intensity of ammonia is measured to assess inhibition of TG2 (Benjakul and Visessanguan, 2003).

From the result of the ammonia assay, it is evident that there is an increase in the intensity of fluorescence with increasing concentrations of the ammonium ions from 0 μM to 1 μM. The points plotted are in accordance with the linear regression model indicating a linear decrease in inhibition of TG2 up to 1 μM concentration of ammonium ions. However, when the concentration of ammonium ions was varied between 0 μM to 0.01 μM, a non-linear curve was obtained indicating that the fluorescence intensity was not directly proportional to the concentration of ammonium ions. When the ammonium ion concentration was varied between 0 μM and 0.1 μM, a linear line was obtained which indicated that increasing concentrations of ammonium ions up to 0.1 μM resulted in a corresponding increase in fluorescence intensity. Ammonia is released during isopeptide bond formation and hence, the rate of release of ammonium ions is a measure of the reaction rate of deamidation by transglutaminase (Skovbjerg et al, 2004).

From the results of the ammonia assay, it can be deduced that concentrations of ammonium ions up to 0.01 μM does not result in any increase in fluorescence intensity. The

intensity of fluorescence starts increasing after the concentration of the ammonium ion goes beyond 0.01 μM up to 1 μM. Hence, in order to achieve any measurable inhibition of TG2 activity, the concentration of ammonium ions need to be 0.01 μM or above.

In order to fine tune the results of the ammonia assay and overcome its limitations, the fluorescence assay was developed to measure the inhibition of TG2 by inhibitors such as putrescine, spermidine, spermine, cadaverine and methylamine. The inhibition activity of putrescine, spermidine, spermine, cadaverine and methylamine on TG2 activity has been well documented in the literature and hence, these were used in the assay to test its sensitivity and reliability (Schaertl et al, 2010; Lentini et al, 2011).

As is evident from the result, considerable inhibition was obtained by methylamine and spermidine, moderate inhibition was obtained by spermine and putrescine, and no visible inhibition was obtained by cadaverine. Transglutaminase inhibitors work by preventing the acidic shift that take place during deamidation and hence, inhibit the reaction. Some others might also act by blocking the active site of the enzyme (Iwai et al, 2014). However, the reading in the control well was very high indicating some point of error in the experiment. This could be one of the reasons why inhibition by cadaverine was not visible in the graph. This leads us to the conclusion that the result of this assay cannot be completely trusted due to an unconventional value of fluorescence intensity in the control well.

Due to inaccurate and unreliable results obtained in the enzyme assay, ammonia assay and fluorescence assay, an electrophoretic assay was developed making use of the deamidation function of TG2 and its inhibition was tested by standard inhibitors. Deamidation is a form of protein modification that takes place in cells where an amide group is replaced by an acid group. It can occur in several amino acids in different proteins and has important physiological

relevance (Hao et al, 2011). One of the activities of TG2 is the deamidation of amino acids in proteins, specifically glutamine which is converted to glutamate (Pinkas et al, 2007). A cysteine residue in the active site of the enzyme catalyzes the reaction and results in the release of ammonia (Stamnaes et al, 2008).

TG2 is a substrate-specific enzyme as it can catalyze deamidation of some substrates and transamidation of other substrates (Dai et al, 2011). The deamidation function of TG2 is usually activated when the concentrations of TG2 or the substrate is limiting or the pH is low (Boros et al, 2006). In this assay, the enzyme was incubated with a storage protein to allow deamidation to occur. The extent of deamidation of the protein and the protein in combination with various inhibitors was tested by running the samples on a non-denaturing PAGE. If deamidation occurred, it would indicate that the activity of TG2 had not been inhibited and vice versa.

From the result obtained, it is apparent that deamidation occurred in wells where the gpl protein was incubated with Ca^{2+} and EDTA (wells H and I) and it did not occur in wells where TG2 or pure TG2 was incubated with Ca^{2+} and EDTA, as the deamidated protein being smaller in size travels faster on a non-denaturing PAGE. This implies that the inhibitors Ca^{2+} and EDTA were able to inhibit the deamidation activity of TG2 and not of the gpl protein. This result is consistent with literature which proves that Ca^{2+} and EDTA can inhibit the deamidation activity of TG2 (Vanbelle et al, 2005).

The deamidation property of transglutaminase works on proteins rich in glutamine and proline residues. As the storage protein was made up of numerous sites where deamidation could occur, it was a suitable substrate for transglutaminase. Polyglutamine stretches or alternating residues of glutamine and proline are highly susceptible to deamidation by transglutaminase. The

storage protein, being rich in these amino acids, underwent deamidation by the enzyme as shown in the figure (Skovbjerg et al, 2004).

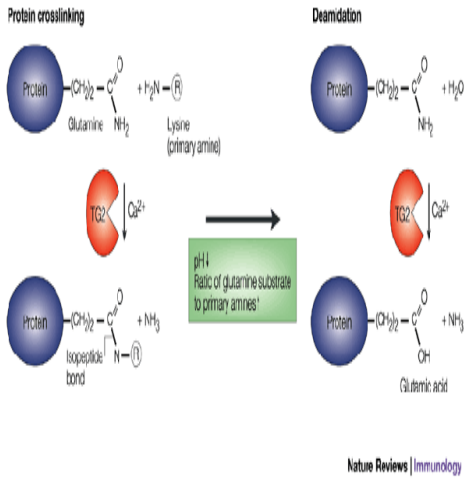


Figure 1: Deamidation of protein by transglutaminase 2 (TG2) (Sollid, 2002).

Using the same approach, the storage protein-enzyme mixture was incubated with other inhibitors of TG2 such as cystamine, ZDON, R283, putrescine, spermine, spermidine, cadaverine, EDTA and water and the extent of deamidation was tested by running the samples on a non-denaturing PAGE (Alcock et al, 2011). As expected, the deamidation reaction was inhibited in all the wells as seen in the result. Deamidation by transglutaminase does not occur when inhibitors are used (Lentini et al, 2011). This indicates that the inhibition of transglutaminase activity by standard inhibitors was picked up by the electrophoretic assay.

Next, the inhibitors Ca^{2+} and EDTA were kept constant and the substrates were changed to measure the inhibition of deamidation activity of transglutaminase on different substrates. The proteins used were Elafin, α-crystallin, β-crystallin and storage protein. Elafin is a protease inhibitor present in the lung and it targets proteinase 3 and leukocyte elastase. It can be cross-linked by transglutaminase which in turn inhibits the action of elafin (Baranger et al, 2011). The

crystallin family of proteins are found in the lens where they function in increasing the refractive power and maintaining transparency. These proteins are also substrates for deamidation by transglutaminases (Asomugha et al, 2010). The objective was to find out if different substrates showed different inhibition profiles on non-denaturing PAGE.

From the result, it is clear that the deamidation of β-crystallin was not inhibited by either Ca^{2+} or EDTA. The deamidation of α-crystallin and storage protein was inhibited by both the inhibitors. Hence, deamidation occurred only for β-crystallin and not for the other proteins. The α-crystallin protein is a simple one with a molecular weight of 19.9 kDa (Horwitz, 2003). On the other hand, the β-crystallin protein is a complex one and can be isolated as a complex having a molecular weight ranging from 46 kDa to 200 kDa (Takata et al, 2009). This could be a possible reason for the deamidation of β-crystallin not being inhibited by Ca^{2+} or EDTA. Also, the elafin protein was not visible on the gel and this could be attributed to the fact that it is a very small protein having a molecular weight of 6 kDa and it could have run out from the gel (Chowdhury et al, 2006). There have been reports which suggest that native elafin is usually undetectable unless conjugated with another protein (Henriksen et al, 2004).

Another assay was designed to understand the disappearance of elafin protein from the gel. In the presence of an elafin inhibitor, the deamidation of elafin by transglutaminase was inhibited in the presence of both Ca^{2+} and EDTA. This result is similar to the wells in which EDTA and Ca^{2+} controls were used indicating that the addition of elafin inhibitor to the mixture allowed inhibition of deamidation to occur. Hence, no deamidation occurred here. As elafin is a protease inhibitor, it is likely that it was interfering with the reaction and when the elafin inhibitor was added to the reaction, deamidation by transglutaminase and inhibition of

deamidation by Ca^{2+} and EDTA could proceed as seen on the gel (Guyot et al, 2008; Galipeau et al, 2014).

CONCLUSION

The TG2 assay, ammonia assay and fluorescence assay were performed to assess the activity of the enzyme TG2. In the TG2 assay, inhibition of TG2 activity occurred at 50% concentration of casein peptides and 125 µM of DMSO. In the fluorescence assay, inhibition was observed only in the case of the inhibitor, methylamine. As all these assays provided negative results, the electrophoretic assay was performed by using non-denaturing PAGE. This assay was able to overcome all the limitations of the previous assays and give an accurate result. However, the procedure can be improved to increase the accuracy and reliability of the assay.

FUTURE DIRECTIONS

The current approaches of measuring transglutaminase activity based on spectrophotometry, fluorometry, and histochemistry have the limitations of being time-consuming and labor intensive. Hence, it is important to keep looking for assays that can be easily performed and highly reliable at the same time. As the enzyme assay, the ammonia assay and the fluorescence assay are not very reliable, the electrophoretic assay should be taken further to develop a reliable source of measuring transglutaminase activity. Also, making it a high-throughput technique can make it feasible to process a number of samples at the same time.

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