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CLONING OF NILE TILAPIA (*Oreochromis niloticus*) TRANSGLUTAMINASE cDNA

Ormruethai Kampong

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CLONING OF NILE TILAPIA (*Oreochromis niloticus*)

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirement for a Master's Degree.

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งานวิจัยนี้ได้ศึกษาคุณลักษณะของยีนทรานสกลูตามิเนสในระดับโมเลกุล โดยการโคลนยีนทรานสกลูตามิเนสจากตับปลานิล ซึ่งผลจากการหาลำดับนิวคลีโอไทด์ของยีนทรานสกลูตามิเนส พบว่ายีนมีขนาด 2,493 หรือ 2,594 นิวคลีโอไทด์ขึ้นอยู่กับขนาดของส่วน 3' UTR ยีนส่วนที่ถูกแปลรหัสมีขนาด 2,091 นิวคลีโอไทด์ ซึ่งแปลรหัสเป็นกรดอะมิโนได้ 696 ตัว จากลำดับของกรดอะมิโนพบว่า มีความเหมือนกับทรานสกลูตามิเนสจาก ปลาจานแดง, ปลาแซลมอล, ปลาม้าลาย และไก่ อยู่ 78%, 59%, 59% และ 41% ตามลำดับ ส่วนตัวเร่ง ประกอบด้วย Cys 272, His 332 และ Asp 355 ซึ่งเหมือนกับทรานสกลูมิเนสจากปลาจานแดง โดยบริเวณจำเพาะของทรานสกลูตามิเนส จากทั้งปลานิล และปลาจานแดงเหมือนกันคือ Cys 272 แต่ทรานสกลูตามิเนสจากปลานิลจะมีความแตกต่างจากปลาจานแดงเล็กน้อยในส่วนปลาย N-terminal และ C-terminal และพบว่าส่วนของ C-terminal ในปลานิลมีกรดอะมิโนเพิ่มขึ้นมา 1 ตัว น้ำหนักโมเลกุลของทรานสกลูตามิเนสจากปลานิลที่ได้จากการคำนวณคือ 78.9 กิโลดาลตัน และมีค่า pI เท่ากับ 6.31

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____
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ORMRUETHAI KAMPONG: CLONING OF NILE TILAPIA (*Oreochromis niloticus*) TRANSGLUTAMINASE cDNA. THESIS ADVISOR : ASST. PROF. MARIENA KETUDAT-CAIRNS, Ph.D. 87 PP. ISBN 974-533-530-4

CLONING/FULL LENGTH cDNA/TRANSGLUTAMINASE

The cDNA encoding transglutaminase from Nile Tilapia (*Oreochromis niloticus*) liver was cloned and sequenced. The cDNA sequence consists of 2,493 or 2,594 nucleotides depend on the 3' UTR. The cDNA encodes an open reading frame of 2,091 nucleotides coding for 696 amino acids. The amino acid sequence of Nile Tilapia liver TGase showed 78%, 59%, 59% and 41% identity with TGase from red sea bream, chum salmon, zebra fish and chicken, respectively. The catalytic triad of Nile Tilapia TGase consists of Cys 272, His 332 and Asp 355 similar to the red sea bream TGase. The putative active site Cys 272 of the enzyme was complete conserved between the two species. The Nile Tilapia TGase had an extension of 1 amino acid at the C-terminal region and some differences in the N-terminal region when compared with red sea bream TGase. The calculated molecular weight of Nile Tilapia TGase is 78.9 kDa with an isoelectric point of 6.31.

School of Biotechnology

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LIST OF ABBREVIATIONS

bp	=	Base pair
°C	=	Degree Celsius
Ca^{2+}	=	Calcium
dNTP	=	Deoxynucleotide triphosphate
G	=	Gram
kDa	=	Kilodalton
Kb	=	Kilobase
$MgCl_2$	=	Magnesium chloride
Min	=	Minute
mL	=	milliliter
mM	=	Millimolar
pI	=	Isoelectric point
RACE	=	Rapid Amplification of cDNA Ends
rpm	=	Revolution per minute
TGase	=	Transglutaminase
Tm	=	Melting temperature
µL	=	Microliter
µM	=	Micromolar

CHAPTER I

INTRODUCTION

Transglutaminase (TGase) is an enzyme that able to catalyze an acyl transfer reaction between primary amines and glutamine residue of protein chain (acyltransfer reaction). TGase has been found in animals, plant and microorganisms. TGase can be categories into several types according to the location. TGase is an important enzyme in food industry, it can be used for several purposes. TGase has plenty of applications especially in food processing. TGase can improve food texture and water holding capacity, increase nutritional value of protein and prepare heat resistant and water-fast film that can be use as edible film packaging material. TGase can also be applied to meat product, fish product, dairy product, soy bean product and wheat product. Guinea pig liver has been the sole source of commercial TGase for decade but it is extremely expensive. The price is about U.S. $80 for one unit. It is not possible to apply this TGase in industrial scale food processing. Recently TGase from *Streptoverticillium mobaraense* has been used in food industries. However, it is still expensive to import. The recent studied of Worratao and Yongsawatdigul (2005) found that Nile Tilapia show the highest TGase activity of 60.8 unit/g sample when compared with other 11 freshwater fishes. TGase from Nile Tilapia might be used in food processing as good as TGase from microorganism, if this TGase can be produced in an industrial scale. The limitation of direct TGase extraction from Nile Tilapia is the low recovery. With molecular biology technology the TGase gene can be clone

and recombinant TGase from Nile Tilapia can be produce to obtain high activity and purity.

CHAPTER II

LITERATURE REVIEW

2.1 TRANSGLUTAMINASE

2.1.1 Transglutaminase reaction

Transglutaminase (E.C.2.3.2.13 Protein glutamine gamma-glutamyl transferase; TGase) which is known as post translation protein remodeling enzyme was first discovered by Waelsch and co-worker more than 40 years ago (Wilhelm, 1996). TGase is an enzyme that able to catalyze an acyl-transfer reaction between primary amines and glutamine residue of protein chain (acyltransfer reaction), as showed in figure 1a. Figure 1b. demonstrated the ε-amino group of lysine residue in protein act as acyl acceptor, ε- (γ glutamyl) lysine bonds are molecularly formed both inter and intra (Koppelman and De jong, 2002). Without primary amines in the reaction system, water can becomes the acyl acceptor and the γ-carboxyamide of glutamine residues are deaminated, becoming glutamic acid residues (Zhu, 1995) as shown in figure 1c.

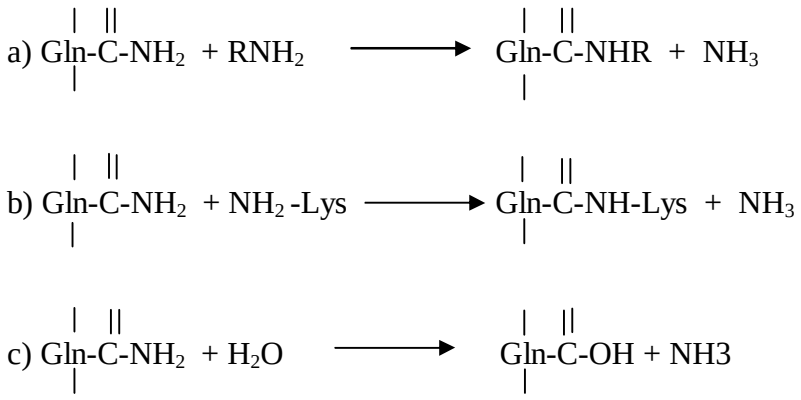


Figure 1 Reaction catalyzed by TGase: (a) acyl transfer reaction; (b) crosslinking reaction between Glutamine and Lysine residues; (c) deamidation reaction.

2.1.2 Type of transglutaminase

TGase has been found in animals, plant, and microorganisms. In 1960s, TGase has been only obtained from animal especially guinea pig livers. Due to its complication of separation and purification processes, other sources have also been investigated later on. In 1989, TGase was found in cultures of *Streptoverticillium* sp. and *Streptomyces* sp. (Nielsen, 1995). TGase can be categorized into several types according to the location. Various TGase's properties have been summarized as follow;

Type 1 TGase; Type 1 TGase found mainly in keratinocyte, it is responsible for cell envelope formation in terminal differentiation of keratinocyte (Griffin, 2002). Keratinocyte TGase which exists in membrane bound and soluble form, is activated several fold by proteolysis. Type 1 TGase are also regulated by several environ- mental factors, especially Ca^{2+} and retionoic acid.

Type 2 TGase; Tissue TGase is closely related to the a subunit of coagulation factor XIII. It is found highly concentrated in liver and also in other tissue. It is a multifunctional molecular player in various cellular processes such as

cell differentiation, matrix stabilization, cancer development, wound healing, cell signaling to apoptosis and pathological conditions such as autoimmune and Huntington diseases (Fesus and Piacentini, 2002). A recent finding in the type 2 TGase field is that the enzyme functions as a signal mediator from receptors to an effectors in transmembrane signaling (Im et al., 1997)

Type 3 TGase; Epidermal TGase which express in many tissues including epidermis, epithelia, brain and placenta. It is also require proteolysis to become active. The function of type 3 TGase concerns cell envelope formation by cross-linking varieties of structural proteins in the epidermis during terminal differentiation.

Type 4 TGase; Prostate TGase is responsible for the reproductive function involving semen coagulation.

Factor XIII; Although all TGases exist in the cytoplasm inside the cells (intracellular TGase), Factor XIII circulates in blood (extra cellular TGase) and works outside cells in the blood coagulation system. Factor XIII found in platelet essential for blood clotting and wound healing. Factor XIII is converted by a thrombin-dependent proteolysis into the active TGase Factor XIII.

Microbial TGase; MTGase have been found in *Physarum polycephalum*, *Streptoverticillium ladakanum* (Lin et al., 2000), *Streptoverticillium mobaraense* and *Bacillus subtilis* (Kobayashi et al., 1996). Unlike many other TGases, MTGase is calcium-independent and has a relatively low molecular weight. Some MTGases have been purified, characterized, cloned and sequenced.

From the entire TGase grouping, the properties of various TGase can be summarized in Table 1.

Table 1. Properties of various TGase

Type of TGase	Source of isolation	Molecular weight (kDa)	Ca^{2+} requirement
Keratinocyte	Human	90	Yes
	Soybean leaves	80	No
Tissue	Guinea pig	76	Yes
	Horseshoe crab	87	Yes
	Human	85	Yes
	Red sea bream	78	Yes
	Nile Tilapia	85	Yes
Epidermal	Human	77	Yes
Prostate	Rat	65	Yes
	Human	77	Yes
Factor XIII	Human	360	Yes
Microbial	*Streptoverticillum*	37.8	No

2.1.3 Characteristic of transglutaminase

1. TGase produces strong binding force. The covalent bond catalyzed by TGase is hard to rupture under the conditions of non-enzymatic reactions. Once the TGase-catalyzed meat is shaped, it will never disperse even when treated with very low temperature (frozen), slicing up or cooking**.**

2. TGase have wide pH stability. Although the optimum pH of crude TGase is 6.0, it also functions with high activity in the range of pH 5.0 to pH 8.0.

3. TGase have high thermal-stability. The optimum temperature of crude TGase is about 50 °C. It also has high activity in the range of 45-55^{o}C.

2.1.4 Transglutaminase applications

From the TGase characteristic, it can be applied in several fields. TGase has plenty of advantages especially in food processing such as;

1. Improvement of the food texture: TGase can improve the important properties of proteins by catalyzing the formation of cross-links of intra and inter molecule of proteins. If TGase is applied in the production of reformed meat, it can not only attach the meat together, but also attach the non-meat protein to meat protein with cross-links, thus greatly improving the taste, flavor, texture and nutritional value of meat products.

2. Improvement of the nutritional value of protein: TGase can make the essential amino acid (such as lysine) covalently cross-linked with protein to prevent the amino acids from being destroyed by Maillard reaction, which results in the improvement of the nutrition value of protein, because covalently incorporated amino acids or peptides behave like amino acid residues in a protein. TGase can also be used to introduce the absent amino acids into protein with unideal compositions.

3. Preparation of heat-resistant and water-fast film: When the TGase-catalyzed casein is dehydrated; a water- insoluble film is obtained. This film can be hydrolyzed by chymotrypsin. Therefore, it is an edible film that can be used as food packaging material.

4. Improving the flexibility water-holding ability of food.

5. Gelation of food protein: Many food proteins can be gelled upon incubating with TGase. The proteins that are not gelled by heating can be gelled. The gels that normally melt at elevated temperature no longer melt after the TGase gelation. The protein in oil-in-water emulsions, even in the presence of sugars and/or

sodium chloride, can be gelled. The gel firmness can be increased after heating. The gels can no longer be solubilized by detergents or denaturants. These indicate that the enzyme is very useful in protein texturization.

The TGase enzyme has been applied to various protein-based products, such as meat, fish, dairy, and vegetables (Nielsen, 1995). The overview applications of TGase in food processing are shown in table 2

Table 2. Overview applications of TGase in food processing (Zhu et al., 1995)

Source	Product	Effect	Reference
Meat	Hamburger, meatballs Frozen meat	Improve elasticity, texture , taste and flavor Improve texture and reduce cost	Sakamoto and Soeda (1991) Takagaki and Narukawa (1990)
Fish	Fish paste	Improve texture and appearance	Ichihara et al. (1990)
Collagens	Shark-fin imitation	Imitation of delicious food	Tani et al. (1990)
Wheat	Baked foods	Improve texture and high volume	Ashikawa et al. (1990)
Soya bean	Tofu	Improve texture and shelf-life	Nonaka et al. (1990)
Vegetables and fruits	Celery	Food preservation	Takagaki et al. (1991)

Table 2. Continued

Source	Product	Effect	Reference
Gelatin	Sweet foods	Low-calorie foods with good texture, firmness and elasticity	Yamanaka and Sakai (1992)
Plant proteins	Protein powders	Gel formation with good texture and taste	Soeda et al.(1992)
Seasonings	Seasonings	Improve taste and flavor	Kobata et al. (1990)
Milk	Yoghurt	Improves the water holding capacity of the gel	Lorenzen (2002)

Here are examples illustrate some of the practical industrial uses of TGase in food processing.

Meat product; TGase has been found to crosslink the proteins within the meat itself, such as fibrin and actin, myosin and actin. TGase can produce restructured meat by binding meat pieces at the present of salt or sodium caseinate (Kennedy, 1999). TGase can be used in combination with fibrinogen and thrombin in producing restructured meat from pieces of meat at a temperature below 10 °C with an overnight reaction time (Pardekooper, 1987). Meat treated with TGase become viscous and viscous caseinate acts as a glue to hold different foodstuffs together. Mince meat and other food ingredients mixed with TGase, shape, packed in pressure-resistant containers and retorted to manufacture meat product. By using this system, a larger piece of restructured meat like beef steaks or fish fillets can be prepared from

their smaller pieces (Motoki and Seguro, 1998). Meat pieces, including minced meat, can be also bound together by TGase without salt (sodium chloride) and phosphates, resulting in healthy meat products.

Fish product; TGase has powerful applications in fish products especially in surimi production. Surimi in Japanese means "Minced Fish", it has been known in asia dietary long time ago. Surimi is a raw material for meat ball, sausage, crab meat, crab imitation, scallop imitation and shrimp imitation. The effect of endogenous TGases extracted from fish species on the setting of various actomyosin paste (Alaska pollock, sardine, common horse mackerel, flounder and Spanish mackerel) were studied by Nowsad et al. (1995). The result showed that TGase promoted the cross-linking of myosin heavy chains during its setting. Actomyosin gel strength measured from breaking force increased with the addition of extracted TGase. The effect of added MTGase at a low temperature setting of Alaska pollock was also studied by Lee et al. (1997). The result obtained from this study supported the previous study. With an increase in setting time and constant quantity of MTGase, gel strength is improved. The higher MTGase, the greater the gel strength. Also, gel strength correlated with increasing amounts of non-disulfide polymerization and ε-(γ-Glu)-Lys dipeptide content. Furthermore a combination of TGase injection and tumbling results in reduced loss during thawing and cooking in frozen fish products.

Dairy product; Many researchers have shown that milk casein, which has no capability of gel formation even by heating, was a very good substrate for various TGases. When TGase is added to untreated milk, only casein was cross-linked and whey protein was left. One of the applications of TGase in dairy product is in yoghurt production. Yoghurt is a milk gel formed by acidic fermentation with lactic starter,

but it may suffer from problems of serum separation with changes in temperatures or physical impacts. This can be solved by adding TGase, because TGase improves the water holding capacity of the gel. Lorenzen and coworker's (2002) found that TGase treatment of milk led to the reduction of mesh sizes of the protein network, and a more regular distribution of the proteins in the yoghurt gel. As a result, yoghurt produced from TGase-treated milk has higher gel strength and less deterioration, especially when the enzyme was not inactivated (Lorenzen et al., 2002). Another dairy product which TGase has a great potential is cheese making. TGase catalyzed incorporation of whey β-lactoglobulin and α-lactalbumin into the cheese curd lead to the production of novel cheese. Cozzolino et al. (2003) incubated pasteurized cow milk with TGase. The result from SDS–PAGE showed that TGase decrease both casein and whey protein and increase high-molecular mass of polymer (Cozzolino et al., 2003).

Soy bean product; Soya proteins, such as 11S and 7S globulins are good substrates for the TGase reaction. Tofu, a typical soybean curd product that is very difficult to produce long-life tofu, since the soft, smooth texture of tofu is easily destroyed by retort sterilization. With the potential of TGase, it is able to maintain the texture of retorted tofu for longer time (Motoki and Seguro, 1998).

Wheat product; In wheat products, noodles and pasta treated with TGase prevented deterioration in textures after cooking even when low-grade flours were used (Sakamoto, 1996). The sensory quality of pastry is favorably affected by the addition of TGase. For the pan breads, it has been observed that the higher specific volume of bread, low hardness values and higher sensory score for visual and texture attributes to the addition of TGase (Collar et al., 2004). The TNO Nutrition and Food Research found that pasta and bread dough prepared in the presence of TGase obtain

higher quality of dough (Bauer et al., 2003). Moreover, the resistance of the dough increased and the extensibility decreased. During the baking process, the TGase-containing dough was less sticky and thus better for the machine (TNO Nutrition and Food Research, 2005).

Vegetables and fruits; Takagaki et al. (1991) reported a method for coating vegetables and fruits with TGase and protein for preservation. Freshness of vegetables and fruits is maintained by coating with membrane containing TGase and proteins. Cut celery treated with an aqueous solution containing TGase, protein, gelatins and Partner-S (natural bacteria) and heated at 50 °C for 5 min to form coating membranes. The coating celery kept at 20 °C for 3 days showed 300 bacterial cell /g, compared to 2 x 10^6 without treatment.

2.1.5 Source of transglutaminase in industrial uses

There are three approaches to developing industrial TGase. The first approach is to extract and purify the enzyme from the tissues or body fluids of food-use animals, such as cattle, swine, and fish such as cod, salmon and flounder. In Europe, factor XIII, a certain type of TGase is extracted commercially from the blood of cattle and swine from slaughter house. However, the blood enzyme is rarely utilized in food manufacture, since thrombin (a specific protease) is required to activate the enzyme and the red pigmentation often damage the product appearance. The second approach is to screen for TGase producing microorganisms. If an appropriate microorganism which produces TGase could be found, it would be possible to mass produce TGase by traditional fermentation technology (Zhu et al., 1995). The third approach is to obtain the enzyme from genetic manipulation by

using host microorganisms, such as *Escherichia coli, Bacillus,* yeast, and *Aspergillus*. Many researchers, including Ikura et al., (1988) express the guinea pig liver TGase in *E. coli*. Bishop et al., (1990) produced human factor XIIIa in yeast. Takehana et al., (1994) express *Streptoverticillium* TGase. in *E. coli*. Washizu et al., (1994) used *Streptoverticillium* TGase in *Streptomyces* sp., and Yasueda et al., (1995) clone and express fish TGase in *E. coli.* All of these cloning and expression have been done in attempt to obtain large amount of TGase at low price.

2.1.6 Substrate specificity

Ajinomoto U.S.A., Inc. has demonstrated the specificity of the TGase enzyme to various types of the protein substrates (table 3). Meat proteins, gelatin and actin are very good substrates in reacting with the enzyme because of their random structure. The 11S globulin and 7S globulin in soybean, casein and sodium caseinate in milk are also good substrates due to their high lysine content and glutamine residues. The conformation of the substrate is another major factor affecting the enzyme activity. (Available:http://scholar.lib.vt.edu/theses/available/etd12219811024 3/unrestricted/ suklim2.PDF)

Table 3. Reactivity of Transglutaminase on various proteins.

Available:http://scholar.lib.vt.edu/theses/available/etd122198110243/ unrestricted/ suklim2.PDF)

Source of protein	Protein reaction
Milk Casein	very well
Na-caseinate	very well
α -lactalbumin	depending on conditions
β-Lactoglobulin	depending on conditions
Egg white protein (ovalbumin)	depending on conditions
Egg yolk Protein	well
Meats Myoglobin	depending on conditions
Collagen	well
Gelatin very	well
Myofibril: myosin	very well
Myofibril: actin	mostly does not react
Soybean 11S globulin	very well
7S globulin	very well
Glutenin	well

2.1.7 History of transglutaminase cloning

In 1995, Yasueda and group cloned cDNA encoding TGase from red sea bream liver. The cDNA sequences consist of 695 amino acids and showed 43 % identity to the sequence of guinea pig liver TGase. The red sea bream TGase gene

was expressed in *E. coli.* and the TGase activity was investigated using the incorporation of monodansylcadaverine into N, N' dimethyl casein. The result showed that recombinant *E. coli* cultured at 37°C produced only inactive TGase while active TGase was found when cultured at 28°C (Yasueda et al., 1995).

In 1996, Sano and his colleague isolated cDNA clones encoding TGase from salmon (*Onchorhynchus keta*). The cDNA sequence has an open reading frame coding a protein of 680 amino acids. The sequence showed high similarity (62.4%) to the red sea bream TGase and the overall structure of salmon TGase resembles the tissue-type TGase, with has some unique structures (Sano et al., 1996).

In 1997, Ikura and team constructed an expression plasmid containing the cDNA of guinea pig liver and transform into *E. coli*. The result showed that recombinant TGase did not indicate significantly different in specific activity when compared with the natural TGase. The sensitivity to activation by Ca^{2+} and rate of catalyzed protein cross linking of recombinant TGase were similar to the natural TGase (Tsuchiya et al., 1997).

In 1998, Duran and colleague purified, characterized and clone the gene of TGase from *Streptoverticillium cinnamoneum* CBS 683.68. The purified enzyme had a relative molecular weight of 37.6 kDa determined by mass spectrophotometry and contain a single Cys residue that was essential for catalytic activity. Contrast to the eukaryotic TGase, this enzyme is Ca^{2+} independent. *S. cinnamoneum* CBS 683.68 TGase contained 411 amino acid residues corresponding to signal peptide of 81 amino acids and mature TGase of 330 amino acid residues. Amino acid analysis shows that *S. cinnamoneum* CBS 683.68 TGase had very little sequence homology with eukaryotic TGase, but share high identity to *Streptoverticillium* strain S-8112 TGase

(Duran et al.,1998).

In 1998, Katsunori and teams cloned and express TGase from *B. subtilis* in *E. coli*. The *tgl* gene, encoding TGase in *B. subtilis* contained 735 nucleotides that encode 245 amino acids residues with molecular weight of 28.3 kDa. The amino acid sequence had little sequence similarity with other TGase from the *Streptoverticillium* sp. or mammals. TGase activity was detected in *E. coli* cells transformed with the plasmid for expression of the *tgl* gene (Katsunori et al., 1998)

In 2002, Villabobos and team successfully cloned and characterized maize TGase. In these studied two related cDNA clones (*TGZ15* and *TGZ21*) encoding maize chloroplast TGase were identified. TGase cDNA from *TGZ15* clone consist of 1,748 bp, which translate to 534 amino acids of 60.9 kDa. and the cDNA from *TGZ21* clone consist of 1,910 bp, which translate to 588 amino acids of 67 kDa. These TGase genes were expressed in *E. coli* and the TGase activities encoded by both maize cDNA clones were detected in bacterial extract. The result demonstrated that the lyses extract from *E. coli* cell transfected with phage containing insert of maize cDNA encode active TGase (Villalobos et al., 2004).

In 2003, Chih-Cheng Huang and his colleague cloned and characterized Tiger shrimp TGase. Tiger shrimp TGase had the highest similarity to crayfish TGase and exhibited significant similarity with other invertebrate TGase and members of the vertebrate TGase gene family. The TGase cDNA consist of 2,988 bp of 757 amino acids with molecular mass of 84.7 kDa. They cloned the Tiger shrimp TGase into Sf21 cell with Lipofectin the result showed that recombinant TGase have the enzyme activity but lacked coagulation activity (Hauang et al., 2004).

In 2005, Worratao and Yongsawatdigul found that Nile Tilapia showed the

highest TGase activity of 60.8 unit/g sample when compared with 11 other freshwater fishes (Worratao and Yongsawatdigul, 2005). However, they characterized the Nile Tilapia TGase only at the biochemical level but not at molecular level. Table 4 shows the summary of TGase cloning from various sources and their results.

Table 4. Summarize of TGase cloning from various sources and their results

Organism	Amino acid	Molecular Weight (kDa)	Host	Result	Reference
Redsea bream liver	695	78.0	*E. coli*	Active TGase was produce at 28°C	Yasueda, et al.,1995
Salmon liver	680	-	-	Overall structure similar to tissue type TGase	Sano, et al.,1996
Guinea pig liver	691	76.6	*E. coli*	Specific activity was not different from natural TGase	Ikura, et al.,1997
Streptoverticillium cinnamoneum CBS 683.68.	411	37.6	-	High identity to *Streptoverticillium* strain S-8112.	Duran, et al.,1998
Bacillus subtilis	245	28.3	*E. coli*	Active TGase was detected in *E.coli* cells	Katsunori, 1998

Table 4. Continued

Organism	Amino acid	Molecular Weight (kDa)	Host	Result	Reference
Maize - *TGZ15*	534	60.9	*E. coli*	Both clones	Villabobos,
- *TGZ21*	588	67.0	*E. coli*	produced active enzyme	et al., 2002
Tiger shrimp	757	84.7	Sf21 cell	active enzyme but lack coagulation activity	Hauang, et al.,2004
Nile Tilapia muscle	-	85	-	TGase activity highest when compare with other 11 freshwater fishs	Worratao, 2005

Symbol (-) mean not studied

2.1.8 Molecular characteristic of transglutaminase

Factor XIII

Factor XIII is a glycoprotein that circulates in blood. Plasma factor XIII structure has been solved at 2.8 A° resolution by x-ray crystallography. It is a heterotetramer composed of two A subunits and two B subunits.

The A chain factor XIII is activated by thrombin and calcium ion to a transglutaminase that catalyzes the formation of gamma-glutamyl-epsilon-lysine

cross-links between fibrin chains, thus stabilizing the fibrin clot, also cross-link alpha-2-plasmin inhibitor, or fibronectin, to the alpha chains of fibrin. Factor XIIIa consist of 730 amino acid residues with MW of 83 kDa.

(Available: http://harvester.embl.de/harvester/P004/P00488.htm.)

The B chain of factor XIII is not catalytically active, but is thought to stabilize the A subunits and regulate the rate of transglutaminase formation by thrombin (Ichinose, 1986). Factor XIIIb consist of 661 amino acid residues with MW of 75 kDa. (Available: http://harvester.embl.de/harvester/P051/P05160.htm)

Tissue type TGase

The complete amino acid sequence of guinea pig liver TGases, a typical tissue-type TGase, was predicted by the cloning and sequence analysis of DNA complementary to its mRNA. Guinea pig TGase cDNA sequence contained 2,073 bases that encoded 691 amino acids. The molecular weight of guinea pig liver TGase was calculated to be 76.6 kDa from the deduced amino acid sequence (Ikura, 1988).

The crystal structure of the tissue-type TGase from red sea bream liver has been determined at 2.5 A° resolution using the molecular replacement method. The model contains 695 amino acid residues. Red sea bream TGase consists of four domains, its overall and active site structures are similar to those of human factor XIII. However, significant structural differences were observed in both the acyl donor and acyl acceptor binding sites, which account for the difference in substrate preferences (Noguchi et al., 2001)

MTGase

The crystal structure of a MTGase from *Streptoverticillium mobaraense* has been determined at 2.4 A° resolution. The catalytic traid of MTGase consists of

Cys 64, Asp 255, His 274 superimpose well on the catalytic triad "Cys-His-Asp" of the factor XIII-like TGase, in this order. The secondary structure frameworks around these residues are also similar to each other. These results imply that both TGase are related by convergent evolution. However, the MTGase has developed a novel catalytic mechanism specialized for the cross-linking reaction. The structure accounts well for the catalytic mechanism, in which Asp 255 is considered to be enzymatically essential, as well as for the causes of the higher reaction rate, the broader substrate specificity, and the lower deamidation activity of this enzyme.

Molecular characteristic comparison of factor XII, red sea bream and MTGase

Overall structure of red sea bream is similar to factor XIII. Both of them consists of four domains named β-sandwich, core. Barrel 1 and barrel 2 (Yee et al., 1994). On the other hand, MTGase structure contained 11 α-helix and 8 β-strands. Therefore the overall structure is completely different from red sea bream and factor XIII. Furthermore red sea bream and factor XIII required Ca^{2+} for catalytic activity but MTGase does not.

The catalytic traid of factor XIII consists of Cys 314, His 373 and Asp 396 while the catalytic triad of red sea bream consists of Cys 272, His 332 and Asp 355 (Noguchi et al., 2001). The catalytic triad of MTGase consists of Cys 64, Asp 255, and His 274 (Kashiwagi et al., 2002). The arrangements of the secondary structures around the active sites of MTGase and red sea bream TGase are very similar. The active site cysteines, Cys 64 in MTGase and Cys 272 in red sea bream TGase, both exist near the N-terminus of the α-helices (α 3 helix in MTGase). On the other hand,

in the active site of MTGase, there is no such cysteine protease-like catalytic triad as "Cys-His-Asp (Asn)." This situation is the most striking and important difference between MTGase and factor XIII.

Activation Processes of the active sites of red sea bream TGase and factor XIII were compared. Because red sea bream TGase and factor XIII have similar active site structures and both require Ca^{2+} to exhibit their catalytic activity, their activation processes are thought to be similar. The catalytic Cys residue is situated between a Tyr residue (Tyr-515 in red sea bream and Tyr-560 in factor XIII) and another Cys residue (Cys-333 in red sea bream and Cys-374 in factor XIII). The Tyr belongs to barrel 1 and is located over the core, which suppresses the enzymatic activities of the TGases. Therefore, the Tyr must be removed to activate the enzymes. The activation process was described by Noguchi (2001). First, Ca^{2+} bound to the binding site of TGase resulting in the conformation change. Then the Tyr covering the catalytic Cys is removed. The Acyl donor (glutamine) bound with the Cys at the active site resulting in acyl-enzyme intermediate and NH_3 was released. Finally, the acyl aceptor (lysine) accesses the active site resulting in GL bond (Noguchi et al., 2001). The activation process of factor XIII is the same as red sea bream but its need thrombin to cleave the factor XIII to factor XIIIa in the present of Ca^{2+}. On the other hand, the Cys 64 of MTGase is sufficiently exposed to the solvent and can promptly react with substrates. Moreover, the flexibility of the right side wall of the active site cleft may decrease the steric hindrance between the enzyme and substrates. These structural dissimilarities between MTGase and the factor XIII TGase may be the reason for the differences in the substrate specificity and the reaction rate.

2.2 RESEARCH OBJECTIVE

The results of Worratao and Yongsawatdigul (2005) of characterization of Nile Tilapia at the biochemical level found that Nile Tilapia showed the highest TGase activity of 60.8 unit/g sample when compared with 11 other freshwater fishes and crude TGase can catalyzed cross-linking of myosin heavy chain. However they did not characterize the Nile Tilapia TGase at the molecular level. Therefore in this thesis, molecular characterization of Nile Tilapia TGase has been done. By clone the full length Nile Tilapia using multiple sequence alignments of the protein sequence to back translates to design degenerate primer to amplify partial cDNA sequence of Nile Tilapia TGase. Then 3' and 5' RACE were performed to obtain the 3' and 5' products. Several difficulties were found, but finally the 3' and 5' RACE product were cloned and sequence. The sequence of 3' and 5' RACE products were used to design new primers to amplify full length cDNA of the Nile Tilapia TGase. At the end full length cDNA sequence from Nile Tilapia TGase was obtained.

CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 NileTilapia (*Oreochromis niloticus*)

NileTilapia (*Oreochromis niloticus*) was purchased from the Suranaree University of Technology (SUT) farm and transfer to laboratory. The fish was shock on ice for 20 minutes and was killed immediately. RNA from NileTilapia liver was extracted.

3.1.2 Chemicals

All chemicals used were molecular grade or analytical grade.

1. Reagents for RT-PCR amplification.
   - 10X RobusT reaction buffer(Finzymes)
   - 10 mM dNTP mix (Invitrogen)
   - RNase inhibitor (40 U/µl)
   - 50 mM $MgCl_2$ (Invitrogen)
2. Reagents for 3' RACE
   - 5X first strand buffer (Invitrogen)
   - 10 mM dNTP mix (Invitrogen)
   - 0.1 M DTT (Invitrogen)
   - DNase inhibitor (Invitrogen)

- 25 mM $MgCl_2$ (Invitrogen)

3. Reagents for 5’ RACE

   - 10X PCR buffer (200mM Tris-HCl (pH8.4), 500 mM KCl)

   - 25 mM $MgCl_2$ (Invitrogen)

   - 10 mM dNTP mix (Invitrogen)

   - 0.1 M DTT (Invitrogen)

   - 5X Tailing buffer (50mM Tris-HCl (pH8.4), 125 mM KCl, 7.5 mM $MaCl_2$)

   - 2 mM dCTP (Invitrogen)

4. Reagents for agarose gel electrophoresis

   - 0.5 % TAE buffer (Appendix 2.4.1)

   - Agarose low EEO. Molecular biology grade (Research organics)

   - 1 kb Ladder marker DNA (Bio lab)

   - 100 bp Ladder marker DNA (Fermentas)

   - 6X Loading dye (Promega)

   - Staining solution; 0.5 µg/ml ethidium bromide in distilled water

5. Reagent for transformation

   - X-Gal (20mg/ml)

   - IPTG (20% W/V)

   - Ampicillin (100 mg/ml)

6. Reagent for sequencing

   - Big dye reaction mix (Applied Biosystem)

   - Sequencing buffer (Applied Biosystem)

   - 3 M sodium acetate

- Absolute ethanol

- Template Suppression Reagent (TSR)

3.1.3. Media (appendix 1)

- LB broth

- LB agar

- SOC

3.1.4. Vector

- pGEM-T-easy vector (Promega)

3.1.5. Enzyme

- Taq DNA polymerase (Home-made, 5units/µl)

- T4 DNA ligase (Promega)

- SuperScrip III RT (Invitrogen)

- RNase mix (Invitrogen)

- Terminal deoxynucleotidyl transferase ,TdT (Promega)

- M-MuLV RT Rnase H^- (Finnzymes)

- DyNAzyme EXT (Finnzymes)

- *Eco*RI (Fermentas)

- *Pst*I (Promega)

- *Sac*I (Promega)

3.1.6 Primer; Most primers for partial cDNA synthesis were designed from the multiple sequence alignments. The primer for 3' and 5' RACE were designed from the partial cDNA sequence. The full length primers were designed from the 3' and 5' RACE sequence. All primers were ordered from Proligo, Singapore.

Table 5. All primers used in the experiment

Primer name	Sequence	Primer size (bp)	Tm (°C)	GC content (%)	Use for
1TGsae_f	TA(T/C)GGICA(A/G)TG(T/C)TGGGTITT	20	57	-	Partial
2TGase_r	ACIGGI(C/A)(C/A)IGGICC(A/G)CA(A/G)CA	20	62	-	Partial
3TGase_f	TGGAACT(G/T)CGGICAITCGAI	20	54	-	Partial
4TGase_r	TCIGC(A/G)TTIAC(T/C)TCIGC(A/G)AAIAC(A/G)AA	26	49	-	Partial
1NTGsae_f	TACGGGCAGTGTTGGGTGTTT	21	54	52	Partial
4NTGase_f	TTCGTCTTTGCCGAGGTCAACGCCGA	26	63	58	Partial
4NTGase_r	TCGGCGTTGACCTCGGCAAAGACGAA	26	63	58	Partial
11NTGase_r	ACCCTGATCAGGT(T/G)ATGCTCAGA	23	57	52	Partial
5NTGase_r	CATGCAACAGCAGCAAACACC	22	68	54	5'RACE
7NTGase_f	GATCCCACCCCTCAAGAA	18	56	55	3'RACE
8NTGase_f	GGAGTGAAGTACGATGCTCC	20	62	55	3'RACE
9NTGase_f	TGGAATTTCCACTGCTGGGTGGAG	24	74	54	3'RACE
10NTGase_r	CTCCACCCAGCAGTGGAAATTCCA	24	74	54	5'RACE
12NTGase_r	GGCGTGTTGGGATTCCCAG	19	62	63	5'RACE
15NTGase_r	GCACTGTACATGCAACAGCAGCAAACACCC	30	69	53	5'RACE

Table 5. Continued

Primer name	Sequence	Primer size (bp)	Tm (°C)	GC content (%)	Use for
16NTGase_r	GGTGATGAGGCGTG(G/A)TGG GATTCCCAGAC	29	71	58	5'RACE
17NTGase_r	CAGATGTCCATCACATAGTC CTC	23	68	47	5'RACE
18NTGase_r	GCATCGTTGGAGTTATTCAGG	27	82	52	5'RACE
19NTGase_r	CTCTGCTCAATGTCCATCTGT GAG	24	72	50	5'RACE
21NTGase_f	GGAGCAGATATTTACGATGG CCAACCAC	28	61	50	Full length
22NTGase_r	GTCCACCAGCAGCTTCCTCA CCCC	24	64	67	Full length
Qt	CCAGTGAGCAGAGTGACGAG GACTCGAGCTCAAGCTTTTTT TTTTTTTTTT	52	69	40	3'RACE
Qo	CCAGTGAGCAGAGTGACG	18	53	61	3'RACE
Qi	GAGGACTCGAGCTCAAGC	18	53	61	3'RACE
AAP	GGCCACGCGTCGACTAGTAC GGGIIGGGIIGGGIIG	36			5'RACE
AUAP	GGCCACGCGTCGACTAGTAC	20	58	65	5'RACE
T7_f	TAATACGACTCACTATAGGG AGA	23	53	39	Sequence
SP6_r	CATACGATTTAGGTGACACT ATAG	24	52	38	Sequence

3.2 METHODS

3.2.1 Primer designed for partial cDNA synthesis

Primers were designed from the Blastp and Blastn (NCBI) results using TGase from red sea bream as a query sequence search. The results of Blastp and Blastn analysis showed amino acids and nucleotide sequence from various TGase sources. Primers were designed by multiple alignments of amino acids and nucleotide sequence of TGase from various sources. Conserve regions of amino acid sequence were chosen for primer designing then compared with the nucleotide sequence. The conservation between both amino acids and nucleotide sequence were used to design degenerate or specific primers.

3.2.2 Total RNA extraction

Total RNA from NileTilapia liver was extracted by using NucleoSpin RNA II Kit (Machery nagel). Briefly, disrupt up to 30 mg of tissue with liquid nitrogen. Add 350 µl buffer RA1 and 3.5 µl ß-mercaptoethanol to the ground tissue and vortex vigorously. Reduce viscosity and clear the lysate by filtration through Nucleospin filter units by place the Nucleospin filter units in collection tube, apply the mixture, and centrifuge for 1 minute at 11,000 x g. Discard the Nucleospin filter units and add 350 µl of 70 % ethanol to the homogenized lysate and mix by vortexing. Take one NucleoSpin RNA II column placed in a 2 ml centrifuge tube and load the lysate. Centrifuge for 30 seconds at 8,000 x g., place the column in a new collection tube then add 350 µl MDB (Membrane Desalting Buffer) and centrifuge at 11,000 x g for 1 minute to dry the membrane. Apply 95 µl DNase reaction mixtures directly onto

the center of the silica membrane of the column. Incubate at room temperature for 15 minutes. Wash and dry silica membrane, first wash add 200 µl buffer RA2 to the NucleoSpin RNA II column, centrifuge for 30 seconds at 8,000 x g then place the column into a new collecting tube. Second wash, add 600 µl buffer RA3 to the NucleoSpin RNA II column, centrifuge for 30 seconds at 8,000 x g then discard flow-through and place the column back into the collecting tube. Third wash, add 250 µl buffer RA3 to the NucleoSpin RNA II column, centrifuge for 2 minutes at 11,000 x g to dry the membrane completely then place the column into a nuclease-free 1.5 ml microcentrifuge tube. Elute the RNA in 60 µl H_2O (RNase-free) and centrifuge at 11,000 x g for 1 minute. The RNA are then kept at – 70 °C till used.

3.2.3 Partial cDNA synthesis

Total RNA from NileTilapia liver was used as a template for one step RT-PCR using RobusT II RT-PCR kit (Finnzymes). Briefly, each 50 µl RT-PCR reaction consisted of 10X Robust reaction buffer, 50 mM $MgCl_2$, 10mM each deoxynucleotide triphosphate (dNTP), 20U of RNase inhibitor, template RNA, 10 µM 1NTGase_f primer, 10 µM 11NTGase_r primer, 10U of M-MuLV RT Rnase H^-, 2U of DyNAzyme EXT and RNase free water. The mixture was incubated at 50 °C for 60 minutes then inactivated M-MuLV RT RNase H^- activity and denature cDNA/RNA hybrid at 94 °C for 2 minutes. The amplification condition were 35 cycles of denaturation 94 °C for 30 seconds, annealing 50 °C for 30 seconds, extension 72 °C for 1.0 minute, followed by final extension 72 °C for 7 minutes. The amplified RT-

PCR products were used as a template for nested PCR. Briefly, each 25 µl nested PCR reaction consisted of 10X PCR buffer, 50 mM $MgCl_2$, 10 mM dNTP mix, 10

µM 1NTGase_f primer, 10 µM 4NTGase_r primer, RT-PCR product, sterilized distilled water and 2.5U of Taq DNA polymerase. The nested PCR amplification condition were 35 cycles of denaturation 94 ℃ for 30 seconds, annealing 60 ℃ for 30 seconds, extension 72 ℃ for 45 seconds, with a 94 ℃ for 5 minutes pre run and 72 ℃ for 7 minutes post run. Amplified DNA product was separated on 1.3 % agarose gel in 0.5X TAE buffer at 100V for 40 minutes, stained with ethidium bromide and visualized under UV light. The PCR products were extracted from the gel, purified, cloned and sequenced.

3.2.4 3’ End cDNA synthesis

3’ RACE is a technique for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and an unknown sequence at the 3' end of the mRNA. 3’ RACE takes advantage of the natural poly (A) tail in the mRNA as a generic priming site for PCR amplification. In this procedure, mRNAs are converted into cDNA using reverse transcriptase (RT) and an oligo-dT adapter primer (AP). Specific cDNA is then directly amplified by PCR using a gene-specific primer (GSP) that anneals to a region of known sequences and an adapter primer (UAP or AUAP) that targets the poly (A) tail region. The 3´ RACE procedure is illustrated in figure 2.

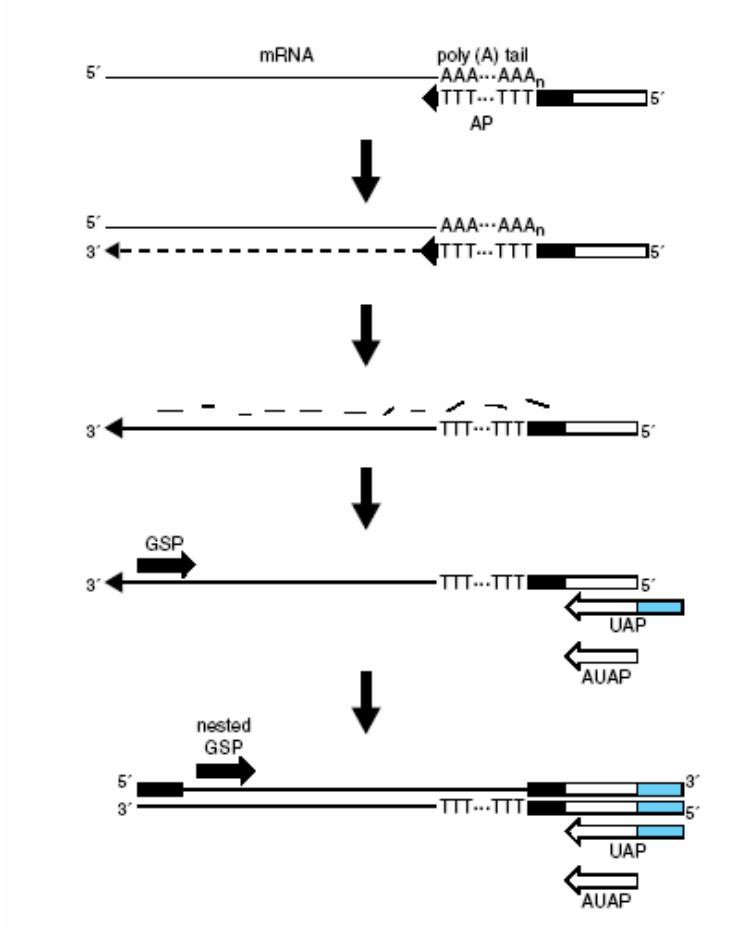


Figure 2 Summary of the 3´ RACE system procedure.

3' End cDNA synthesis procedure

3' End cDNA synthesis by 3' RACE method, each 20 µl first strand cDNA synthesis reaction consisted of total RNA, 10 mM dNTP, 100 µM Qt primer. Heat the mixture to 65°C for 5 minute and chill on ice for 2-3 minutes. The following content were added to the tube, 5X first strand buffer, 0.1 M DTT, DNase inhibitor and SuperScript™ III RT. The tube was incubated at 55°C in heat block for 60 min and terminates the reaction by incubated at 70°C for 15 minutes. The target cDNA were amplified by PCR. The reaction contained 10X PCR buffer, 50 mM $MgCl_2$, 10 mM dNTP mix, 10 µM 9NTGase_f primer, 10 µM Qo primer, distilled water and

Taq DNA polymerase. The PCR amplification condition were 35 cycles of denaturation 94 °C for 30 seconds, annealing 53 °C for 30 seconds, extension 72 °C for 90 seconds, with a 94 °C for 5 minutes pre run and 72 °C for 7 minutes post run. The first strand cDNA products were used as a template for nested PCR. Briefly, each 25 µl nested PCR reaction consisted of 10X PCR buffer, 50 mM $MgCl_2$, 10 mM dNTP mix, 10 µM 8NTGase_f primer, 10 µM Qi primer, first strand cDNA product, sterilized distilled water and 2.5U of Taq DNA polymerase. The nested PCR amplification condition were 35 cycles of denaturation 94 °C for 30 seconds, annealing 60 °C for 30 seconds, extension 72 °C for 90 seconds, with a 94 °C for 5 minutes pre run and 72 °C for 7 minutes post run. Amplified DNA product were separate on 1.3 % agarose gel in 0.5X TAE buffer at 100V for 40 minutes, stained with ethidium bromide and visualized under UV light. The PCR products were extracted from the gel, purified, clone and sequence.

3.2.5 5' End cDNA synthesis and cloning

5' RACE is a technique for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and an unknown sequence at the 5' end of the mRNA. First strand cDNA synthesis is primed using a gene-specific antisense oligonucleotide (GSP1). This permits cDNA conversion of specific mRNA. Following cDNA synthesis, the first strand product is purified from unincorporated dNTPs and GSP1 primer. Terminal deoxynucleotidyl transferase (TdT) is used to add homopolymeric tails to the 3' ends of the cDNA. In the original protocol, tailed cDNA is then amplified by PCR using a mixture of primers: a nested gene-specific primer (GSP2), which anneals 3' to GSP1 and a combination of a complementary

homopolymer-containing anchor primer and corresponding adapter primer which permit amplification from the homopolymeric tail. This allows amplification of unknown sequences between the GSP2 and the 5’ end of the mRNA. The 5’ RACE procedure is illustrated in figure 3.

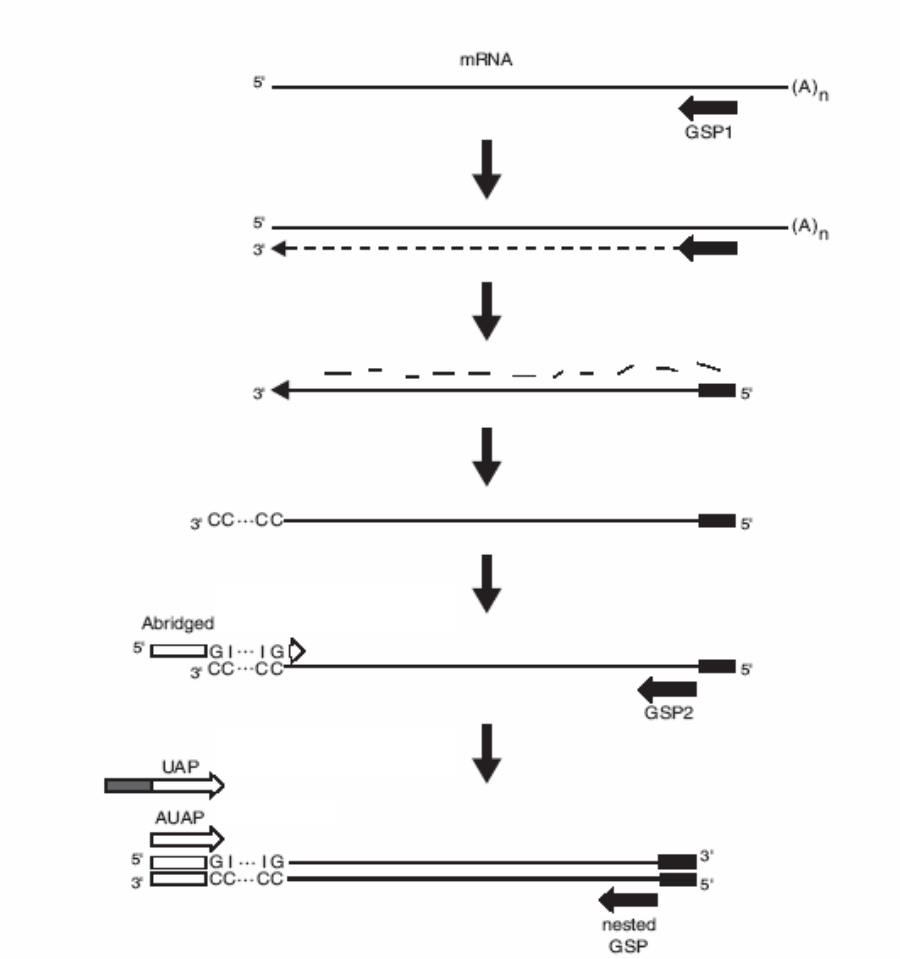


Figure 3 Summary of the 5’ RACE system procedure.

5’ End cDNA synthesis procedure

5’ End cDNA synthesis by 5’ RACE method was not to get full 5’ end of the gene, therefore the 5’ RACE was done in 2 steps as followed.

First strand cDNA synthesis

Each 26 µl first strand cDNA synthesis reaction consisted of total RNA**,** 2.5 µM 10NTGase_r or 19NTGase_r, 10X PCR buffer, 25 mM $MgCl_2$, 10 mM dNTP mix, 0.1 M DTT, SuperScript III RT and DEPC-treated water**.** The reaction was incubated for 50 minute at 42 °C. The reaction was terminated at 70 °C for 15 minutes. RNase mix was added to the reaction and incubated for 30 min at 37 °C. The reaction was collected and placed on ice.

S.N.A.P column purification of cDNA

First strand cDNA was purified by using S.N.A.P column (Appendix 2.5)

TdT tailing of cDNA

Each 25 µl consist of 5X tailing buffer, 2 mM dCTP, S.N.A.P. purified cDNA, TdT and DEPC-treated water. The reaction was incubated for 10 minutes at 37 °C. TdT was inactivated for 10 minutes at 65 °C.

PCR of dC-tailed cDNA

The following components were added into tube, 10X PCR buffer, 25 mM $MgCl_2$, 10 mM dNTP mix, 10 µM 16NTGase_r or 17NTGase_r, 10 µM Abridged Anchor Primer (AAP), dC-tailed cDNA, Taq DNA polymerase and sterilized distilled water. The PCR amplification condition was 35 cycles of denaturation 94 °C for 30 seconds, annealing 55 °C for 30 seconds, extension 72 °C for 2 minutes, with a 94 °C for 5 minutes pre run and 72 °C for 7 minutes post run. 5' RACE product was analyzed by agarose gel electrophoresis according to standard protocols.

3.2.6 Full length cDNA synthesis

Full length cDNA of target gene can be generated by one of following methods.

End-to-End PCR

If the extreme sequence of the 5’ and 3’ends of target cDNA are known, design 5’ and 3’ primers for the generation of target full length cDNA. The extreme sequences of the 5’ and 3’ends can be obtained from the 5’ and 3’RACE products of the gene.

Ligation of 5’ and 3’ RACE Fragments

If unique restriction enzyme sites in target cDNA are known, generation of the full length target cDNA can be done. First, generate 5’ and 3’RACE products of target cDNA that should have the overlapping region between them. The unique restriction site should be presented within the overlapping region. Second, the 5’ and 3’ RACE fragments are digested by the unique restriction enzyme and the digested 5’ and 3’ RACE fragments are ligated to generate full-length cDNA.

Direct PCR using 5’ and 3’ RACE Fragments

If the sequence information of the 5’ and 3’ ends of target cDNA was unknown or could not find the unique restriction enzyme site in the overlapping region of the 5’ and 3’ RACE fragments, the full length target cDNA can be generated by direct PCR using the 5’ and 3’ RACE fragments. Full length cDNA of interest can be obtained by two PCR reactions. First PCR reaction is performed using 5’ and 3’ RACE products without adding the primers. The fragments of 5’ and 3’ RACE have two roles: template and primer. The priming site is the overlapping region between 5’ and 3’ RACE fragments. Full length cDNA of interest is synthesized at first PCR

reaction. Second PCR reaction is commenced using first PCR product as a template and 5' and 3' RACE primers. Full length cDNA of interest is amplified at second PCR reaction.

Full length cDNA synthesis procedure

Total RNA from NileTilapia liver were used as a template for one step RT-PCR by using RobusT II RT-PCR kit. Briefly, each 50 µl RT-PCR reaction consisted of 10X Robust reaction buffer, 50 mM $MgCl_2$, 10mM each dNTP, 20U of RNase inhibitor, template RNA, 10 µM 20NTGase_f primer, 10 µM 14NTGase_r primer, 10U of M-MuLV RT RNase H^-, 2U of DyNAzyme EXT and RNase free water. The mixture was incubated at 50 ° C for 60 minutes then inactivate M-MuLV RT Rnase H^- activity and denature cDNA/RNA hybrid at 94 °C for 2 minutes. The amplification condition was 35 cycles of denaturation 94 °C for 30 seconds, annealing 51 °C for 30 seconds, extension 72 °C for 3.5 minutes, followed by final extension 72 °C for 7 minutes. Amplified DNA product was separated on 1.3 % agarose gel in 0.5X TAE buffer at 100V for 40 minutes, stained with ethidium bromide and visualized under UV light.

3.2.7 Nucleotide sequence

Purified DNA was sequenced each 10 µl contained 1 µl 10X sequencing buffer, 2 µl Big dye reaction mix, 3.2 µM forward or reverse primer, 5 µl plasmid template and 1 µl H_2O. The amplification condition was 25 cycles of denaturation 96 °C for 10seconds, annealing 50 °C for 5 seconds, extension 60 °C for 2.0 minutes, with a 96 °C for 5 minutes pre run. The amplified products were purified by ethanol

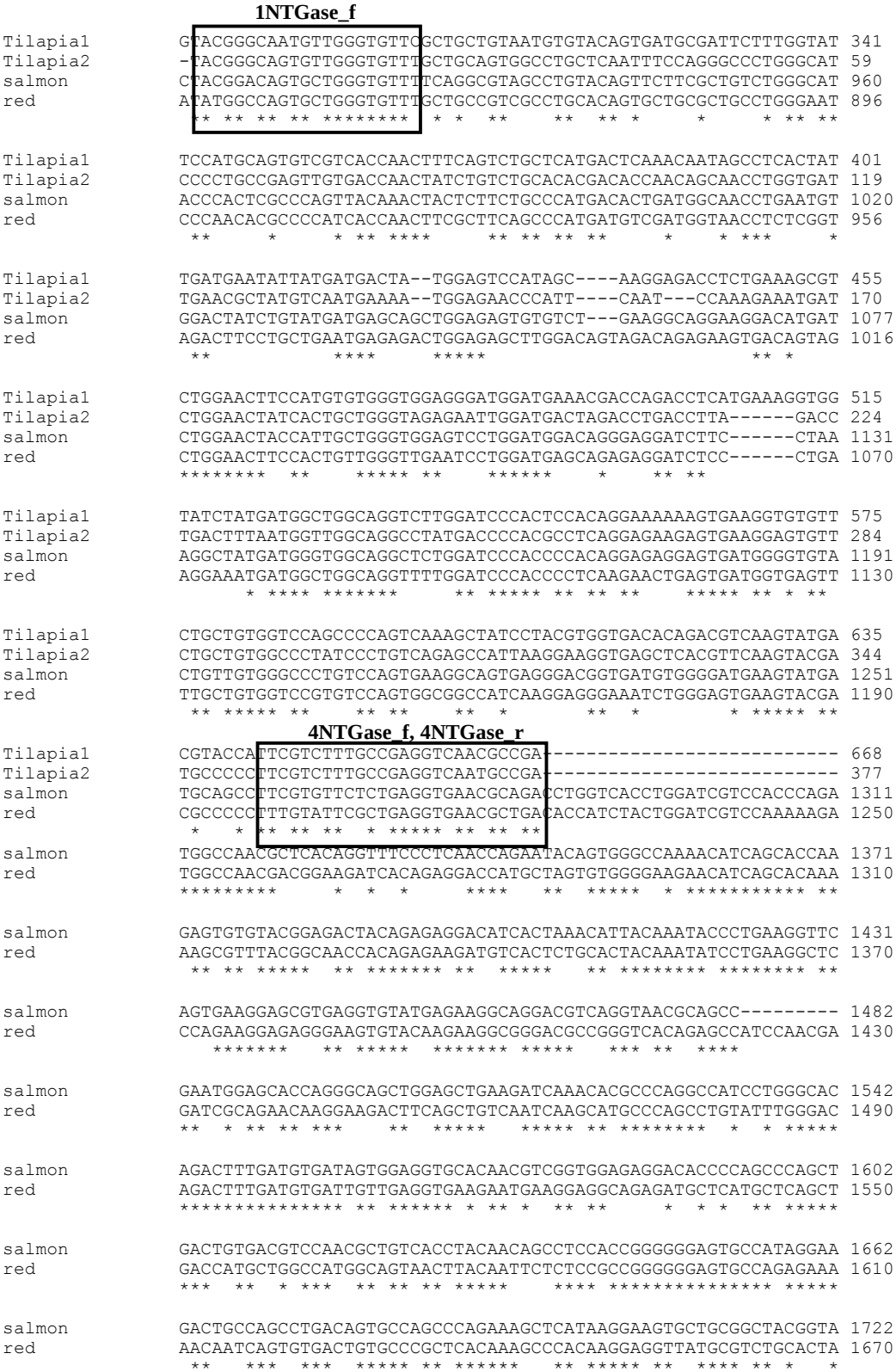
precipitation. The reaction of 3 µl 3 M sodium acetate, 17 µl H_2O and 60 µl absolute ethanol were add to purified product. Then the reaction was incubated at room temperature for 15 minutes, centrifuge max speed (14,000 rpm) for 20 minutes and discard supernatant. The pellet was washed with 250 µl 70% ethanol, centrifuge max speed for 5 minutes and discard supernatant. Pellet was dried at 90 °C for 1 minute then added 15 µl TSR to dissolve pellet, heated at 95 °C for 2 minutes, quick chilled on ice for 2 minutes.

CHAPTER IV

RESULTS AND DISSCUSIONS

4.1 PRIMER DESIGNED FOR PARTIAL cDNA SYNTHESIS

Primers for cDNA synthesis were designed from various TGase alignments. Amino acid sequences of TGase from six organisms, red sea bream, chicken, salmon, frog, cattle and human were aligned using ClastalW program. From the TGase alignment, four conserve regions were used to back translate and design four degenerate primers (1TGase_f, 2TGase_r, 3TGase_f and 4TGase_r) to amplify partial cDNA sequence of the TGase gene from Nile Tilapia (*O. niloticus*) liver. From the PCR results, the PCR product that relate to the expected size was obtained but the sequence of the fragment were not TGase gene, so the primers were redesigned for specific primers. From Loonchanta and Ketudat-cairns (2004) studied, they obtained partial cDNA of TGase from Nile Tilapia muscle. From the report they obtained 2 groups of partial cDNA sequences. New primers were designed from nucleotide sequence alignment of salmon, red sea bream and the 2 groups of Nile Tilapia muscle sequences. Primer 1NTGase_f was redesigned from 1TGase_f, primer 4NTGase_f and 4NTGase_r were redesigned from 4TGase_r and a new primer 11NTGase_r was designed from salmon and red sea bream alignment.


1NTGase_f
4NTGase_f, 4NTGase_r
Tilapia1 GTACGGGCAATGTTGGGTGTTCGCTGCTGTAATGTGTACAGTGATGCGATTCTTTGGTAT 341
Tilapia2 -TACGGGCAGTGTTGGGTGTTTGCTGCAGTGGCCTGCTCAATTTCCAGGGCCCTGGGCAT 59
salmon CTACGGACAGTGCTGGGTGTTTTCAGGCGTAGCCTGTACAGTTCTTCGCTGTCTGGGCAT 960
red ATATGGCCAGTGCTGGGTGTTTGCTGCCGTCGCCTGCACAGTGCTGCGCTGCCTGGGAAT 896
Tilapia1 TCCATGCAGTGTCGTCACCAACTTTCAGTCTGCTCATGACTCAAACAATAGCCTCACTAT 401
Tilapia2 CCCCTGCCGAGTTGTGACCAACTATCTGTCTGCACACGACACCAACAGCAACCTGGTGAT 119
salmon ACCCACTCGCCCAGTTACAAACTACTCTTCTGCCCATGACACTGATGGCAACCTGAATGT 1020
red CCCAACACGCCCCATCACCAACTTCGCTTCAGCCCATGATGTCGATGGTAACCTCTCGGT 956
Tilapia1 TGATGAATATTATGATGACTA--TGGAGTCCATAGC----AAGGAGACCTCTGAAAGCGT 455
Tilapia2 TGAACGCTATGTCAATGAAAA--TGGAGAACCCATT----CAAT---CCAAAGAAATGAT 170
salmon GGACTATCTGTATGATGAGCAGCTGGAGAGTGTGTCT---GAAGGCAGGAAGGACATGAT 1077
red AGACTTCCTGCTGAATGAGAGACTGGAGAGCTTGGACAGTAGACAGAGAAGTGACAGTAG 1016
Tilapia1 CTGGAACTTCCATGTGTGGGTGGAGGGATGGATGAAACGACCAGACCTCATGAAAGGTGG 515
Tilapia2 CTGGAACTATCACTGCTGGGTAGAGAATTGGATGACTAGACCTGACCTTA------GACC 224
salmon CTGGAACTACCATTGCTGGGTGGAGTCCTGGATGGACAGGGAGGATCTTC------CTAA 1131
red CTGGAACTTCCACTGTTGGGTTGAATCCTGGATGAGCAGAGAGGATCTCC------CTGA 1070
Tilapia1 TATCTATGATGGCTGGCAGGTCTTGGATCCCACTCCACAGGAAAAAAGTGAAGGTGTGTT 575
Tilapia2 TGACTTTAATGGTTGGCAGGCCTATGACCCCACGCCTCAGGAGAAGAGTGAAGGAGTGTT 284
salmon AGGCTATGATGGGTGGCAGGCTCTGGATCCCACCCCACAGGAGAGGAGTGATGGGGTGTA 1191
red AGGAAATGATGGCTGGCAGGTTTTGGATCCCACCCCTCAAGAACTGAGTGATGGTGAGTT 1130
Tilapia1 CTGCTGTGGTCCAGCCCCAGTCAAAGCTATCCTACGTGGTGACACAGACGTCAAGTATGA 635
Tilapia2 CTGCTGTGGCCCTATCCCTGTCAGAGCCATTAAGGAAGGTGAGCTCACGTTCAAGTACGA 344
salmon CTGTTGTGGGCCCTGTCCAGTGAAGGCAGTGAGGGACGGTGATGTGGGGATGAAGTATGA 1251
red TTGCTGTGGTCCGTGTCCAGTGGCGGCCATCAAGGAGGGAAATCTGGGAGTGAAGTACGA 1190
Tilapia1 CGTACCATTCGTCTTTGCCGAGGTCAACGCCGA---------- 668
Tilapia2 TGCCCCCTTCGTCTTTGCCGAGGTCAATGCCGA---------- 377
salmon TGCAGCCTTCGTGTTCTCTGAGGTGAACGCAGACCTGGTCACCTGGATCGTCCACCCAGA 1311
red CGCCCCCTTTGTATTCGCTGAGGTGAACGCTGACACCATCTACTGGATCGTCCAAAAAGA 1250
salmon TGGCCAACGCTCACAGGTTTCCCTCAACCAGAATACAGTGGGCCAAAACATCAGCACCAA 1371
red TGGCCAACGACGGAAGATCACAGAGGACCATGCTAGTGTGGGGAAGAACATCAGCACAAA 1310
salmon GAGTGTGTACGGAGACTACAGAGAGGACATCACTAAACATTACAAATACCCTGAAGGTTC 1431
red AAGCGTTTACGGCAACCACAGAGAAGATGTCACTCTGCACTACAAATATCCTGAAGGCTC 1370
salmon AGTGAAGGAGCGTGAGGTGTATGAGAAGGCAGGACGTCAGGTAACGCAGCC--------- 1482
red CCAGAAGGAGAGGGAAGTGTACAAGAAGGCGGGACGCCGGGTCACAGAGCCATCCAACGA 1430
salmon GAATGGAGCACCAGGGCAGCTGGAGCTGAAGATCAAACACGCCCAGGCCATCCTGGGCAC 1542
red GATCGCAGAACAAGGAAGACTTCAGCTGTCAATCAAGCATGCCCAGCCTGTATTTGGGAC 1490
salmon AGACTTTGATGTGATAGTGGAGGTGCACAACGTCGGTGGAGAGGACACCCCAGCCCAGCT 1602
red AGACTTTGATGTGATTGTTGAGGTGAAGAATGAAGGAGGCAGAGATGCTCATGCTCAGCT 1550
salmon GACTGTGACGTCCAACGCTGTCACCTACAACAGCCTCCACCGGGGGGAGTGCCATAGGAA 1662
red GACCATGCTGGCCATGGCAGTAACTTACAATTCTCTCCGCCGGGGGGAGTGCCAGAGAAA 1610
salmon GACTGCCAGCCTGACAGTGCCAGCCCAGAAAGCTCATAAGGAAGTGCTGCGGCTACGGTA 1722
red AACAATCAGTGTGACTGTGCCCGCTCACAAAGCCCACAAGGAGGTTATGCGTCTGCACTA 1670

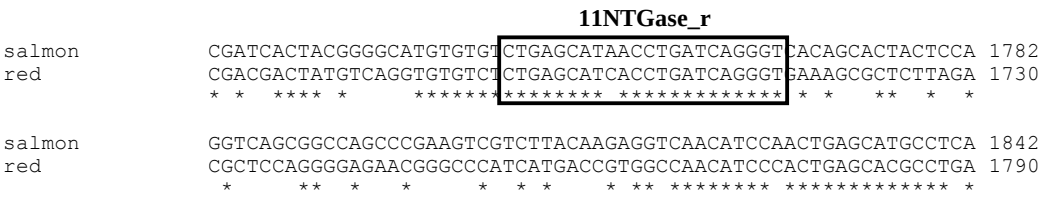


Figure 4 Result of ClustalW alignment from three organisms which are red sea bream, chum salmon and the 2 groups of Nile Tilapia partial cDNA from muscle. Three conserved regions were used to design four specific primers (1NTGase_f, 4NTGase_f, 4NTGase_r and 11NTGase_r) to amplify partial cDNA sequence of TGase gene from Nile Tilapia liver.

4.2 PARTIAL cDNA SYNTHESIS

Total RNA from Nile Tilapia liver was extracted by NucleoSpin RNA II Kit. Total RNA was used as a template for one step RT-PCR reaction. First strand cDNA was synthesized by the reverse transcriptase enzyme with primer 1NTGase_f and 11NTGase_r, finally, second strand cDNA was synthesized. Semi-nested PCR was done by using the RT product as a template with primer 1NTGase_f and 4NTGase_r. And another reaction with primer 4NTGase_f and 11NTGase_r. PCR product was determined by agarose gel electrophoresis. The expected size when amplified with 1NTGase_f and 4NTGase_r, 4NTGase_f and 11NTGase_r should be 387 bp and 618 bp, respectively. The results of the above reactions are shown in figure 5.

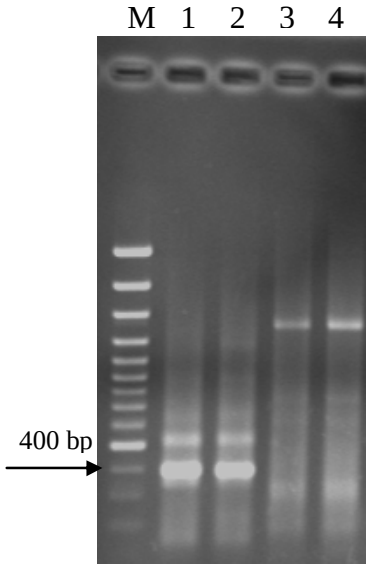


Figure 5 Agarose gel electrophoresis of amplified fragment from PCR reactions with the sets of primers. Lane M: 100 bp marker, lane 1-2: 1NTGase_f and 4NTGase_r, lane 3-4: 4NTGaase_f and 11NTGase_r.

From the PCR results, the major band that related to the expected size was found in lane 1 and 2 (figure 5) which amplified with 1NTGase_f and 4NTGase_r primer. On the other hand, the other primer pairs produce bigger size band than expected, so these band were discard. After that DNA size about 400 bp (major product) from lane 1 and 2 (figure 5) were excised and eluted from agarose gel using DNA extraction II kit (appendix 2.4). The DNA was then ligated into pGEM-T-easy vector and transformed to *E. coli* strain DH5α. The transformants were screened with LB-ampicillin plate contained X-gal and IPTG. All transformants were determined for the TGase inserted by restriction enzyme digestion with *Eco*RI as shown in figure 6.

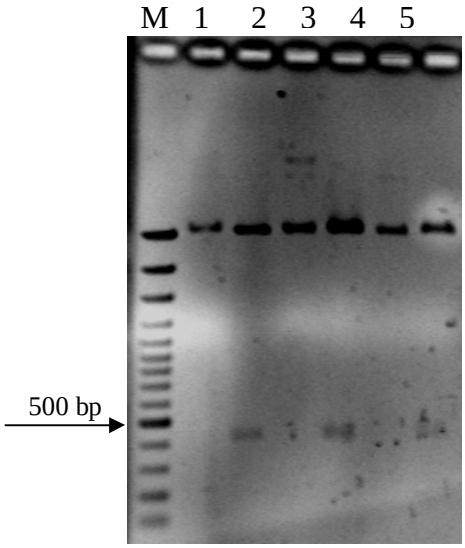


Figure 6 Agarose gel electrophoresis of recombinant plasmid digestion with *Eco*RI. Lane M: 100 bp marker, lane 1, 3, 5: uncut plasmid, lane 2, 4, 6: digested plasmid.

From the digestion results, digested plasmid showed product size about 400 bp. It can be concluded that all 3 clones contained recombinant plasmid that have the partial cDNA of TGase gene. Plasmids from the three positive clones were sequenced using ABI PRISM 310 Genetic Analyzer.

From the sequencing result, all 3 clones showed the same nucleotide sequence, which comprise of 394 nucleotides (figure 7). After partial cDNA sequences were obtained, Blastx analysis was performed. In the Blastx analysis, the nucleotide sequences were translated to 128 amino acids and compared with various proteins in the NCBI database. The result showed that the partial cDNA of Tilapia TGase is most similar to red sea bream TGase with 84 % identity. From the partial cDNA sequencing result, primer for 5' and 3' RACE were designed from the initial and the end of the partial cDNA sequence respectively. The primer's locations on the partial cDNA sequence are showed in figure 7.

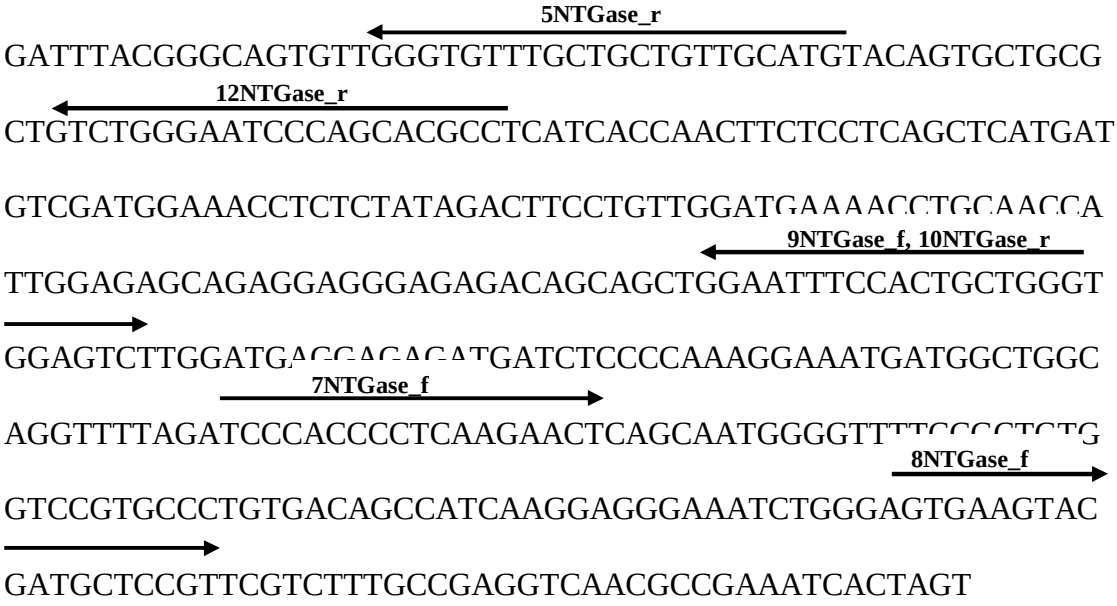


Figure 7 The primer's location on the partial cDNA sequence. Reverse primers were used for 5' RACE and forward primers were used for 3' RACE.

4.3 3' END cDNA SYNTHESIS

3' RACE amplification was done by 2 steps RT-PCR. Total RNA extracted from liver was used as a template for reverse transcription reaction. First strand cDNA was synthesized by reverse transcriptase with primer Qt (poly T primer), which prime to the poly A tail of the mRNA. Then RT products were used as template for the PCR reaction with primers 9NTGase_f and Qo. The PCR product was nested with primer pairs, 7NTGase_f and Qo, 7NTGase_f and Qi, 8NTGase_f and Qo and 8NTGase_f and Qi. The expected size when amplified with 7NTGase_f and Qo, 7NTGase_f and Qi should be approximately 1.4 Kb while amplified with 8NTGase_f and Qo and 8NTGase_f and Qi should be approximately 1.3 Kb. These values were estimated from the red sea bream sequence (AAB35370).

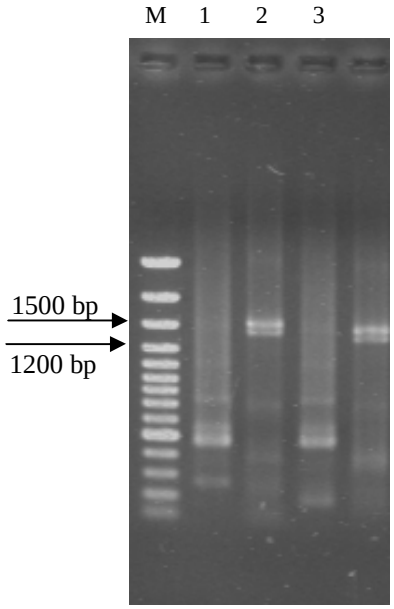


Figure 8 Agarose gel electrophoresis of nested PCR resulted from the sets of primers in amplification of 3' end cDNA sequence of TGase gene. Lane M: 100 bp Marker, lane 1: 7NTGase_f and Qo, lane 2: 7NTGase_f and Qi, lane 3: 8NTGase_f and Qo and lane 4: 8NTGase_f and Qi.

From the gel electrophoresis results (figure 8), the expected size found in the reaction of 7NTGase_f and Qi, 8NTGase_f and Qi (lane 2 and 4). However double bands were observed. The DNA from both upper and lower bands from 7NTGase_f and Qi, 8NTGase_f and Qi were excised and eluted from gel. Then ligated into pGEM-T easy cloning vector and transformed to *E. coli* strain DH5α, plate on LB-ampicillin plate containing X-gal and IPTG. The positive transformants were obtained only from the plate 8NTGase_f and Qi fragment. The positive clones from larger band fragment inserted were determined for the inserted TGase appearance by restriction digestion with *Eco*RI however the result was not clear. Therefore, the plasmids were tested for the 3' RACE TGase insertion by PCR analysis. The plasmids templates were diluted 50 times and 8NTGase_f and Qi were

used as primers. The result in figure 9 showed that lane 3, 4 and 9 contained the appropriate size PCR products that can be concluded that this plasmid should contain the 3' RACE TGase fragments. On the other hand, the clones from larger band fragment inserted were determined for the inserted TGase appearance by restriction digestion with *Eco*RI. The results in figure 10 showed that lane 6 contained the appropriate size. This can be concluded that this plasmid should contain the fragment.

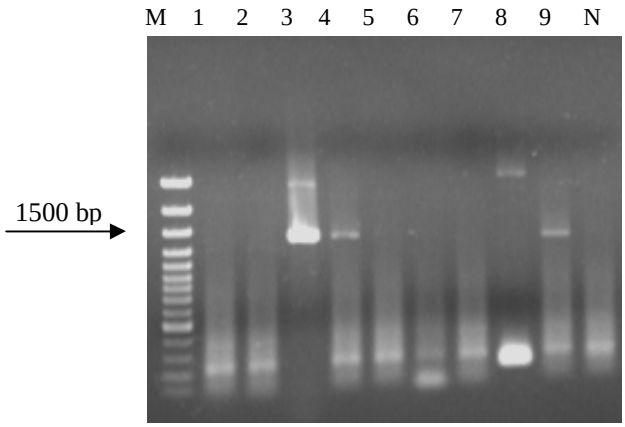


Figure 9 Plasmid amplification by PCR with primers 8NTGase_f and Qi.

Lane M : 100 bp marker, lane 1-9: clone 1-9, laneN: negative control, screen for smaller band.

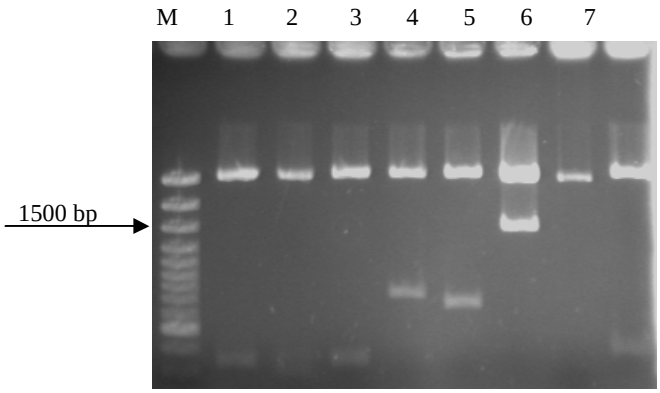


Figure 10 Agarose gel electrophoresis of plasmids digestion with *Eco*RI. Lane M: 100 bp marker, lane1-7: clone 1-7, lane N: negative control, screen for larger band

From the digestion result, clone number 3 from smaller band (lane 3 in figure 9) and clone number 6 from larger band (lane 6 from figure 10) were chosen for nucleotide sequencing. From the sequencing results, both clones showed the same sequence but the larger band had longer 3' UTR sequence than the other. The larger 3' UTR sequence in clone 6 might be due to differences in polyadenylation signal of the 3' ends. Polyadenylation is the addition of poly (A) to the 3' end of mRNA. Transcriptions of eukaryotic genes extend beyond the polyadenylation site. Then the transcript is cleaved and polyadenylated at the 3' end of the cleavage site. Polyadenylation signal is important to polyadenylation process. Polyadenylation signal contain the sequence AAUAAA about 20-30 nucleotide before polyadenylation site. At the RNA level, the sequence AAUAAA occurs in most mammalian mRNA about 20 nucleotides upstream to their poly (A). Although the most common sequence at the RNA level is AAUAAA and it is the most efficient in promoting polyadenylation but the most variant is AUUAAA and it is about 80 % as efficient as AAUAAA. The other variants are much less common, and also much less efficient (Weaver, 2005). Form cDNA sequence of TGase mRNA showed that the TGase mRNA contains 2 polyadenylation signal site at the sequence AUUAAA and AAUAUA as shows in figure 11. Some transcripts were cleaved downstream AUUAAA 20 nucleotides then add poly (A) some other transcript were cleaved downstream AAUAUA 15 nucleotides then add poly (A). The cleavage at downstream AUUAAA gave a shorter product when compare to the cleavage at downstream AAUAUA. The sequences of 3' RACE product were also put in Blastx program. The results showed that 289 amino acids of the 3' end of Tilapia TGase were most similar to red sea bream TGase with 74 % identity.

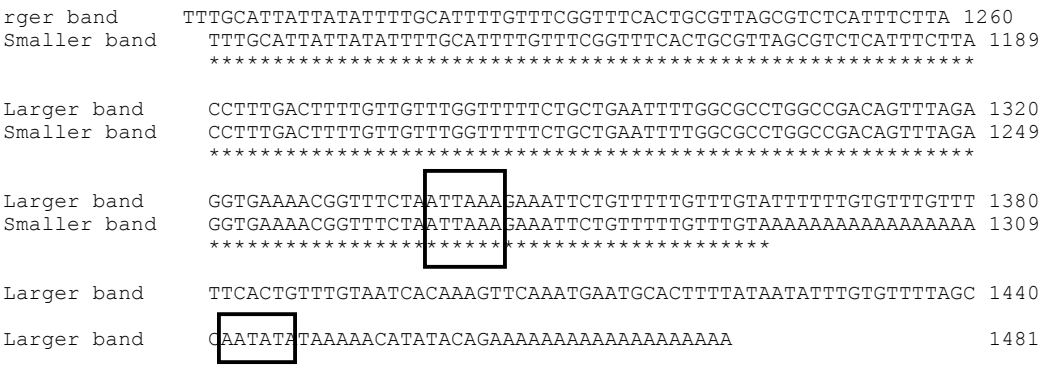


Figure 11 Larger PCR product and smaller PCR product sequence alignment. Open box indicates polyadenylation signal.

4.4 5' END cDNA SYNTHESIS

4.4.1 First 5' End cDNA synthesis

Total RNA was used as a template for 5' end cDNA amplification. First strand cDNA was amplified with primer 10NTGase_r (figure7). Then first strand cDNA was purified by ethanol precipitation and resuspend in DEPC–treated water. Purified first strand cDNA was tailed with dATP by terminal deoxy-nucleotidyltransferase (TdT). Then the cDNA was purified again. Second strand cDNA was amplified with primer Qt. PCR was done using the cDNA as template with primer 12NTGase_r and Qo and then nested with primer 12NTGase_r and Qi, 5NTGase_r and Qo, 5NTGase_r and Qi. The gel electrophoresis result of 5' RACE appears a smear band in all primer pair. This might be due to 3 reasons. First, the mRNA which hybrid to the first strand cDNA was not destroyed resulting in interfering in the next step. Secondly, the DNA that precipitated with ethanol step may not pure enough for PCR amplification or thirdly, the primers are not as specific as expected. So, the method was changed to inverse PCR. Inverse PCR is used to clone sequence flanking a known sequence. Genomic DNA was partial digested and then ligated to

make a circular DNA. PCR primers pointing away from known sequence were used to amplify the flanking unknown sequence. The result of several tries of inverse PCR was not part of the expected TGase cDNA. So, 5' RACE kit was chosen for 5' end cDNA amplification. First strand cDNA were synthesized by superscript III reverse transcriptase with primer 10NTGase_r and total RNA used as a template. After first strand cDNA was synthesized, dNTP was removed from the reaction to prevent the interfering in TdT reaction by S.N.A.P column. Purified first strand cDNA was tailed with dCTP by TdT and used it as a template for PCR amplification with primer 5NTGase_r and AAP, 12NTGase_r and AAP. Then semi nested with 5NTGase_r and AUAP used 12NTGase_r and AAP product as a template. The product size appears approximately 900 bp. Then, DNA was ligated to cloning vector and sequenced. The sequencing result shows that the insert was not TGase gene. This might due to primer non specific to template. So, the primers were designed again for higher Tm and longer nucleotide. Primer 15NTGase_r was redesigned from 5NTGase_r and primer 16NTGase_r was redesigned from 12NTGase_r. PCR amplification was done with primer 15NTGase_r and AAP, 16NTGase_r and AAP. Then, semi nested with primer 15NTGase_r and AUAP, the other reaction with primer 5NTGase_r and AUAP used the template from 16NTGase_r and AAP product. The result of PCR amplification with 15NTGase_r and AAP, 16NTGase_r and AAP and nested PCR product with primer 5NTGase_r and AUAP are shown in figure 12 and 13, respectively.

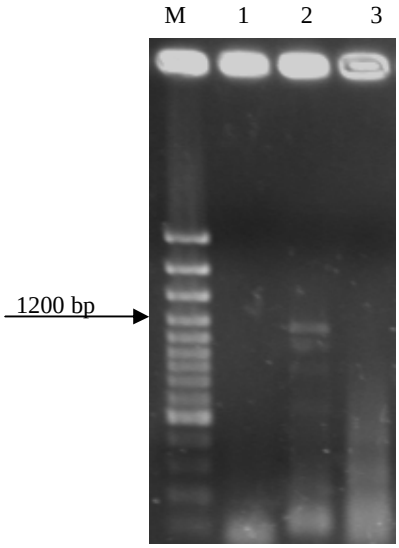


Figure 12 Agarose gel electrophoresis of PCR product resulted from the sets of primers in amplification of 5'end cDNA. Lane M : 100 bp Marker, lane 1: 15NTGase_r and AAP, lane 2: 16NTGase_r and AAP, lane 3: nested PCR product with primer 15NTGase_r and AUAP.

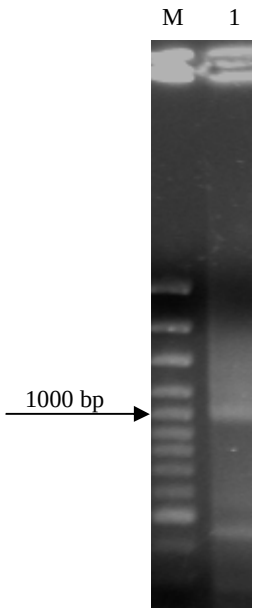


Figure 13 Agarose gel electrophoresis of PCR product resulted from the sets of primers in amplification of 5'end cDNA. Lane M: 100 bp Marker, lane1: nested PCR product with primer 5NTGase_r and AUAP.

From the agarose gel electrophoresis results, the band from 16NTGase_f and AAP, 5NTGase_r and AUAP product showed the sized of 1100 and 1000 bp. Which were bigger than the expected size (approximately 900 bp in red sea bream). Product from gel electrophoresis were excised and eluted, then ligated into cloning vector and transformed to *E. coli* DH5α The positive clones were digests with *Eco*RI to find the insertion of TGase 5' end products. The result of plasmids digestion with *Eco*RI are shown in figure 14 and 15.

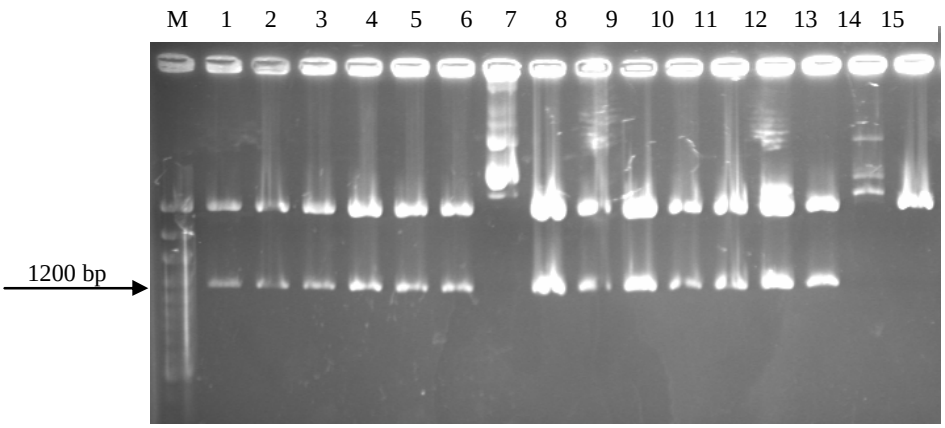


Figure 14 Agarose gel electrophoresis of plasmids digestion with *Eco*RI. Lane M: 100 bp marker, lane1-15: clone 1-15, lane N: negative control, screen for 16NTGase_r and AAP inserted.

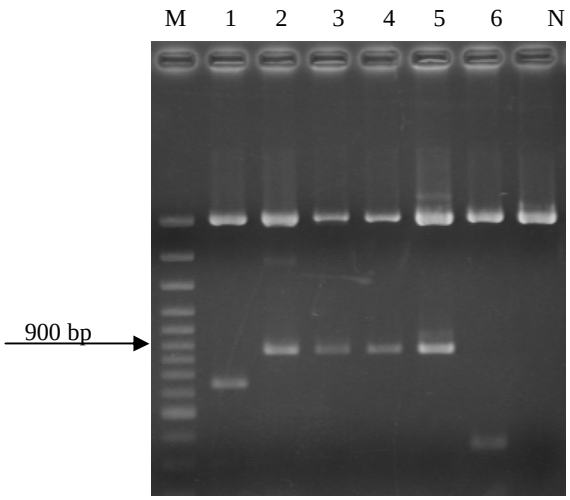


Figure 15 Agarose gel electrophoresis of plasmids digestion with *Eco*RI. Lane M : 100 bp marker, lane1-6: clone 1-6, lane N: negative control, screen for 5NTGase_r and AUAP inserted.

From the digestion results, clone number 10, 12, 13 from 16NTGase_f and AAP amplified (figure 14.) and clone number 4 and 5 from 5NTGase_f and AUAP amplified (figure 15.) were chosen for nucleotide sequencing. From the sequencing results, the nucleotides were then used in the Blastx program. From the Blastx results, showed that clones from 16NTGase_f and AAP amplified were TGase gene but clone from 5NTGase_f and AUAP amplified were not TGase gene. This might be due to 2 reasons, 5NTGase_f has low specificity to the gene or 5NTGase_f and AUAP amplify using a smear band as a template instead of 16NTGase_f and AAP product. Clone number 10, 12, 13 from 16NTGase_f and AAP inserted show the same sequence and 68% identity to red sea bream TGase. But the sequence of 1200 nucleotide was translated to only 90 amino acids of TGase. The rest of sequence were not TGase when compare to red sea bream. And these 90 amino acids did not reach the 5' end of the TGase gene when compare with other mammal and fish TGases.

From the first 5’ RACE sequence, new primers for second 5’ RACE were designed to reach the 5’ end cDNA

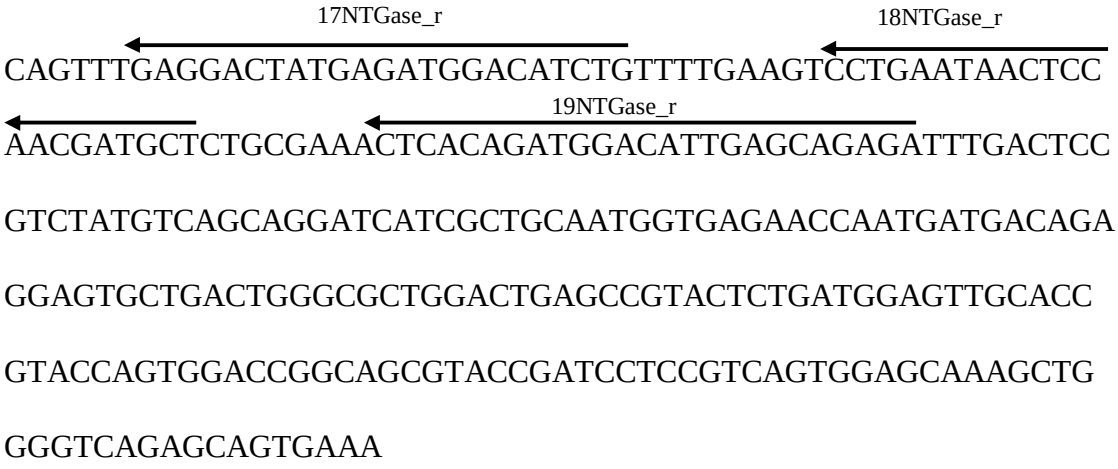


Figure 16 Primer’s location on 5’ end cDNA sequence. Primers 17, 18, 19 NTGase were designed for second 5’ RACE.

4.4.2 Second 5’ End cDNA synthesis

First strand cDNA was synthesized by superscript III reverse transcriptase with primer 19NTGase_r and total RNA used as a template. After first strand cDNA were synthesized, dNTP was removed from the reaction by S.N.A.P column. Purified first strand cDNA was tailed with dCTP by TdT and used it as a template for PCR amplification with primer 17NTGase_r and AAP, 18NTGase_r and AAP. Then semi nested with 17NTGase_r and AUAP was done using 18NTGase_r and AAP product as a template. The expected size is about 700 bp which was estimated from the red sea bream TGase sequence. The PCR amplification result shows in figure 17.

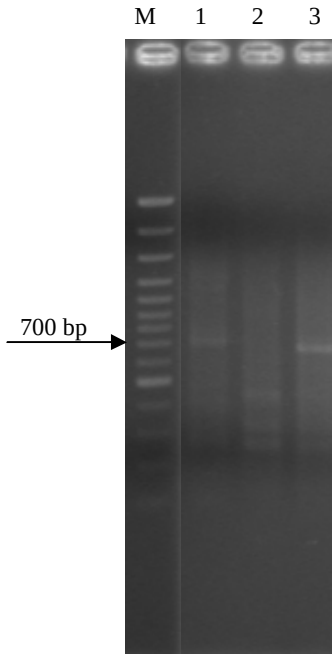


Figure 17 Agarose gel electrophoresis of PCR product resulted from the sets of primers in amplification of 5'end cDNA. Lane M: 100 bp marker, lane1: PCR product with primer 17NTGase_r and AAP, lane2: PCR product with primer 18NTGase_r and AAP, lane3: nested PCR product with 17NTGase_r and AUAP.

The product size appears approximately 700 bp in lane 1 and 3 that amplified with primer 17NTGase_r and AAP, 17NTGase_r and AUAP respectively. Then, DNA was excised and eluted from gel. DNA was then ligated to cloning vector and transform to *E. coli*. Positive clones were determined for the inserted TGase appearance with *Eco*RI digestion. The digestion result shown in figure 18.

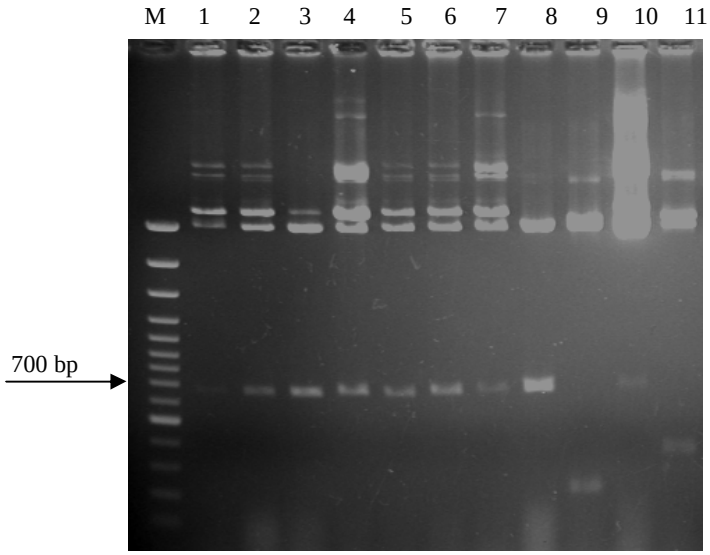


Figure 18 Agarose gel electrophoresis of plasmids digestion with *Eco*RI. Lane M: 100 bp marker, lane1-3: clone 1-3 that amplified with 17NTGase_r and AAP, lane 4-10: clone 4-10 that amplified with 17NTGase_r and AUAP, lane N: negative control.

From the digestion result, clone number 2, 3 from 17NTGase_f and AAP amplified and clone number 7, 8 from 17NTGase_f and AUAP amplified were chosen for nucleotide sequencing. From the sequencing results, the nucleotides were then used in the Blastx program. From Blastx result, clones from 17NTGase_f and AAP amplified were TGase gene but clones from 17NTGase_f and AUAP amplified were not TGase gene. It might be due to 17NTGase_f and AUAP amplified product used a smear band as template so, the nested PCR product was not TGase. Clone number 2, 3 from 17NTGase_f and AAP amplified, show the same sequence and 78% identity to red sea bream TGase.

4.5 FULL LENGTH cDNA SYNTHESIS

Primers for full length cDNA were designed from the 3' and the 5' end cDNA sequences. Total RNA was used as a template for one step RT-PCR reaction. First strand cDNA was synthesized by reverse transcriptase enzyme with 2 primer pairs 20NTGase_f and 14NTGase_r, 21NTGase_f and 14NTGase_r. Semi-nested PCR was done by used RT product from 20NTGase_f and 14NTGase_r amplified as a template with primer 21NTGase_f and 14NTGase_r and other reaction with 21NTGase_f and 13NTGase_r. The expected size should be 2,300 bp when amplified with 20 or 21NTGase_f and 14NTGase_r, and 2100 bp when amplified with 21NTGase_f and 13NTGase_r. The PCR amplification result shows in figure 19.

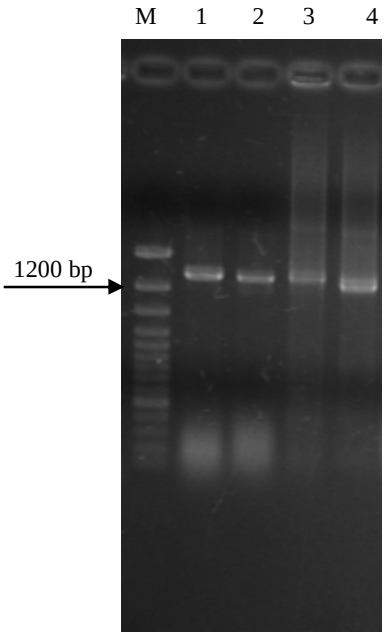


Figure 19 Agarose gel electrophoresis of PCR product resulted from the sets of primers in amplification of 5'end cDNA. Lane M: 100 bp marker, lane 1: PCR product with primer 20NTGase_f and 14NTGase_r, lane 2: PCR product with primer 21NTGase_r and 14NTGase_r, lane3: nested PCR

product with 21NTGase_f and 14NTGase_r, lane 4: nested PCR product with 21NTGase_f and 13NTGase_r.

From agarose gel electrophoresis result, the experimental product size appears relate to the expected size. Primer 20NTGase_f and 14NTGase_r were chosen for amplify full length cDNA. DNA was excised and eluted from gel then ligate into pGEM-T-easy vector and transformed to *E. coli*. Positive clones were determined for the inserted TGase appearance with restriction digestion. From the partial, the 3' and the 5' end cDNA sequence the restriction enzyme digestion map of Tilapia TGase was constructed by Web cutter 2 program. The map showed, *Bam*HI, *Pst*I, *Sac*I and *Hind*III site on Tilapia TGase gene. *Pst*I and *Sac*I were chosen for plasmid digestion. *Pst*I digest 2 sites (174, 1462) on the TGase gene and 1 site on pGEM-T easy vector. The digestion result shows in figure 20.

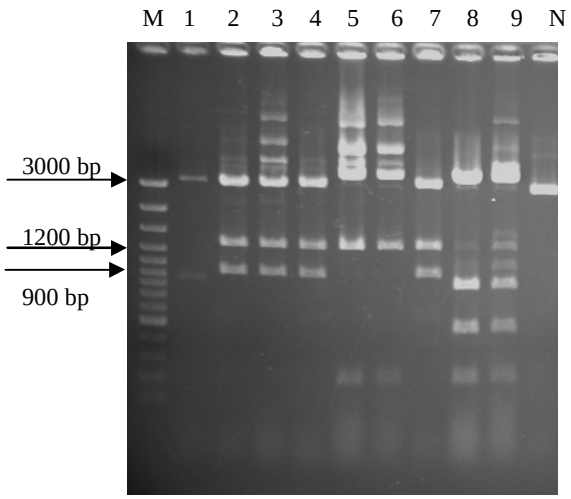


Figure 20 Agarose gel electrophoresis of plasmids digestion with *Pst*I. Lane M: 100 bp marker, lane1-9: clone 1-9, lane N: negative control.

From plasmid digestion with *Pst*I result, digested plasmid was divided to 3 groups. First group, the TGase gene was ligated to pGEM-T easy vector in correct direction (5'end-3'end). The product sizes about 3.17, 1.28 and 0.9 Kb. appeared in clone 2, 3, 4 and 7. The second group, the TGase gene was ligated to pGEM-T easy vector in the opposite direction (3'end-5'end). The product sizes about 3.9, 1.2, and 0.1 Kb. which appeared in clone 5, 6. The last group appears product more 3 bands in clone 8, 9. It might be due to both clone had 1 additional *Pst*I restriction site in the insert. Product size about 1200 bp in both clones may due to incomplete digestion.

Plasmids were digested with *Sac*I, which digest only 1 site (1443) on TGase gene and 1 site on pGEM-T easy vector. The digestion result shows in figure 21.

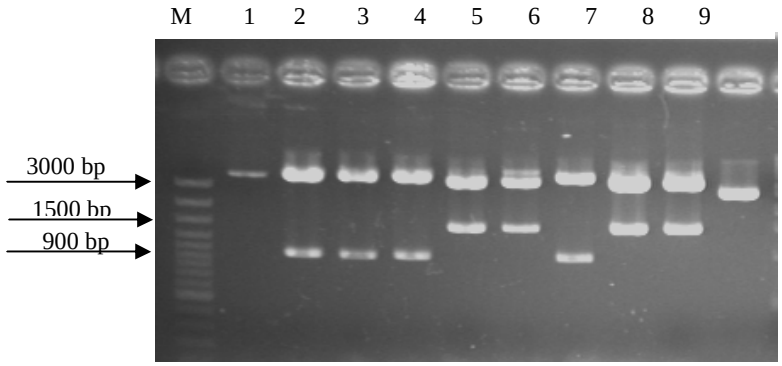


Figure 21 Agarose gel electrophoresis of plasmids digestion with *Sac*I. Lane M: 100 bp marker, lane1-9: clone 1-9, lane10: negative control.

From plasmids digestion with *Sac*I result, digested plasmids were divided to 2 groups. First group, the TGase gene was ligated to pGEM-T easy vector in correct direction (5'end-3'end). The product sizes about 4.4 and 0.9 Kb. were appearing in clone 2, 3, 4 and 7. The second group, the TGase gene was ligated into

pGEM-T easy vector in opposite direction (3'end-5'end). The product sizes about 3.9 and 1.4 Kb. were appearing in clone 5, 6, 8and 9. From the digestion result with both enzymes, most clone except clone 1 contain TGase gene but difference in the insertion direction. Clone 2, 3, 4, 6, 7, 8 and 9 were chosen for sequencing with primer T7, SP6, 4NTGase_r and 9NTGase_f.

4.6 NUCLEOTIDE AND DEDUCED AMINO ACID SEQUENCE OF cDNA

The complete nucleotide sequence of the cDNA insert from pGEM-T-easy vector was determined and found to contain an open reading frame of 2,091 nucleotides. The first ATG of open reading frame was at a nucleotide 45-47. The cDNA contained 361 or 462 nucleotides 3' untranslated regions including the poly A tail and 44 nucleotide 5' untranslated regions. Cloning of the full length cDNA was done at 2 separate times (different RT-PCR reaction). The sequence of the full length TGase from different clones from both PCR amplifications was identical. This indicates that the TGase cloned and sequence did not contain any mutation from PCR error. The deduced amino acid sequence of the open reading frame corresponded to a protein of 696 amino acids. The catalytic triad of Nile Tilapia consists of Cys 272, His 332 and Asp 355 the same as red sea bream as show in figure 24. The putative active site Cys 272 of the enzyme was completely conserved between the two species. The calculated molecular weight of Nile Tilapia TGase is approximately about 78.9 kDa with an isoelectric point of 6.31 (Compute pI/Mw program Available: http:// www.expasy.org/cgibin /pi_tool) compare to the native Nile Tilapia TGase which the molecular weight is 85 kDa with an isoelectric point of 6.53 (Worratao, 2005) while red sea bream TGase had molecular weight of 78.2 kDa (Yasueda, 1995). Since this

is the first Nile Tilapia TGase clone and sequence, therefore the nucleotide sequences were submitted to GenBank and obtain the accession number QD480532. The nucleotide sequence of Nile Tilapia TGase cDNA and deduce amino acid sequence that was submitted to GenBank are shown in figure 22. The primers used in this study are all shown in figure 23.

```
DNA: ACGAATCTGACACAGCGTGAAGACATTTGGAGCAGATATTTACGATGGCCA   51
                                                M  A  N    3

DNA: ACCACAAAGGTTTGATTAGTGATGTGGATCTCAGAAGTCGCGAGAACAACT  102
       H  K  G  L  I  S  D  V  D  L  R  S  R  E  N  N  S   20

DNA: CGGCACACCACACCAGGGAGATCGATCAAAAGCGTTTGATTGTCCGTAGGG  153
       A  H  H  T  R  E  I  D  Q  K  R  L  I  V  R  R  G   37

DNA: GTCAGCCCTTCTCCATCACTCTGCAGTGTAATGGCTCTTTGCGCCCCAGAC  204
       Q  P  F  S  I  T  L  Q  C  N  G  S  L  R  P  R  H   54

DNA: ACCACTTGGATCTGGTCCTGCACCTCGGTAAGAGAGACGAGGTGGAGATCA  255
       H  L  D  L  V  L  H  L  G  K  R  D  E  V  E  I  K   71

DNA: AGGTTCAGAAGGAGCGTGGAGCTGGGGACAAGTGGTGGTTTAACCAGCAGG  306
       V  Q  K  E  R  G  A  G  D  K  W  W  F  N  Q  Q  G   88

DNA: GAGCACAAGATGAAATGCTGCTGACTCTGCACAGTCCAGCTGATGCTATAA  357
       A  Q  D  E  M  L  L  T  L  H  S  P  A  D  A  I  I  105

DNA: TTGGCCGGTACAGTCTGGCTGTGCTGCTGATGTCACCGGACGGACGCATTT  408
       G  R  Y  S  L  A  V  L  L  M  S  P  D  G  R  I  L  122

DNA: TAGAGAAAAAGGACAAAATGAGTTTCCACCTGCTCTATAACCCCTGGTGCA  459
       E  K  K  D  K  M  S  F  H  L  L  Y  N  P  W  C  K  139

DNA: AAGATGATGTGGTGTACCTGCCCGACGAGACGCAGCTTCAGGAGTACATCA  510
       D  D  V  V  Y  L  P  D  E  T  Q  L  Q  E  Y  I  M  156

DNA: TGAACGAAGACGGAATAATCTACATGGGGACCTGGGAATACATCAAAAGCA  561
       N  E  D  G  I  I  Y  M  G  T  W  E  Y  I  K  S  T  173

DNA: CTCACTGGAATTATGGACAGTTTGAGGACTATGTGATGGACATCTGTTTTG  612
       H  W  N  Y  G  Q  F  E  D  Y  V  M  D  I  C  F  E  190

DNA: AAGTCCTGAATAACTCCAACGATGCTCTGCGAAACTCACAGATGGACATTG  663
       V  L  N  N  S  N  D  A  L  R  N  S  Q  M  D  I  E  207
```

```
DNA: AGCAGAGATTTGACCCCGTCTATGTCAGCAGGATCATCGCTGCAATGGTGA  714
       Q  R  F  D  P  V  Y  V  S  R  I  I  A  A  M  V  N  224

DNA: ACTCTAATGATGACAGAGGAGTGCTGACTGGGCGCTGGACTGAGCCGTACT  765
       S  N  D  D  R  G  V  L  T  G  R  W  T  E  P  Y  S  241

DNA: CTGATGGAGTTGCACCGTACCAGTGGACCGGCAGCGTACCGATCCTCCGTC  816
       D  G  V  A  P  Y  Q  W  T  G  S  V  P  I  L  R  Q  258

DNA: AGTGGAGCAAAGCTGGGGTCAGAGCAGTGAAATATGGCCAGTGCTGGGTGT  867
       W  S  K  A  G  V  R  A  V  K  Y  G  Q  C  W  V  F  275
                                          ▲

DNA: TTGCTGCTGTTGCATGTACAGTGCTGCGCTGTCTGGGAATCCCAACACGCC  918
       A  A  V  A  C  T  V  L  R  C  L  G  I  P  T  R  L  292

DNA: TCATCACCAACTTCTCCTCAGCTCATGATGTCGATGGAAACCCCTCTATAG  969
       I  T  N  F  S  S  A  H  D  V  D  G  N  P  S  I  D  309

DNA: ACTTCCTGTTGGATGAAAACCTGCAACCATTGGAGAGCAGAGGAGGGAGAG 1020
       F  L  L  D  E  N  L  Q  P  L  E  S  R  G  G  R  D  326

DNA: ACAGCAGCTGGAATTTCCACTGCTGGGTGGAGTCTTGGATGAGGAGAGATG 1071
       S  S  W  N  F  H  C  W  V  E  S  W  M  R  R  D  D  343
                   ▲

DNA: ATCTCCCCAAAGGAAATGATGGCTGGCAGGTTTTAGATCCCACCCCTCAAG 1122
       L  P  K  G  N  D  G  W  Q  V  L  D  P  T  P  Q  E  360
                                       ▲

DNA: AACTCAGCAATGGGGTTTTCCGCTGTGGTCCGTGCCCTGTGACAGCCATCA 1173
       L  S  N  G  V  F  R  C  G  P  C  P  V  T  A  I  K  377

DNA: AGGAGGGAAATCTGGGAGTGAAGTACGATGCTCCGTTTGTGTTTGCAGAGG 1224
       E  G  N  L  G  V  K  Y  D  A  P  F  V  F  A  E  V  394

DNA: TGAACGCTGACATCATCCACTGGATCGTTCAGAGAAATGGCCAACGGAGAA 1275
       N  A  D  I  I  H  W  I  V  Q  R  N  G  Q  R  R  K  411

DNA: AAATCAGAGTGGACCATGCCACTGTGGGTAGGAACATCAGCACCAAAAGTG 1326
       I  R  V  D  H  A  T  V  G  R  N  I  S  T  K  S  V  428

DNA: TTTATGGGGACTTCAGAGATGATGTCACTCTGCACTACAAATATCCTGAAG 1377
       Y  G  D  F  R  D  D  V  T  L  H  Y  K  Y  P  E  G  445

DNA: GATCCAAGAAGGAGAGAGAAGTGTACGAGAAGGCGGGGCGTCGGGTGACAG 1428
       S  K  K  E  R  E  V  Y  E  K  A  G  R  R  V  T  D  462

DNA: ATGTCCCCAGTGAGAGCTCAGAACCAGGACGACTGCAGCTGTCCATCAAGC 1479
       V  P  S  E  S  S  E  P  G  R  L  Q  L  S  I  K  H  479

DNA: ATGCCAAGCCTGTGTTTGGGACAGACTTTGATGTGATTATTGAGGTGAAGA 1530
       A  K  P  V  F  G  T  D  F  D  V  I  I  E  V  K  N  496
```

```
DNA: ATGAAGGAGACCAGGATGCTCATGTTCAGCTGACCATGCTGGCTATGGCTG 1581
       E  G  D  Q  D  A  H  V  Q  L  T  M  L  A  M  A  V  513

DNA: TTACTTACAGCTCTCTTCATCGGGGGAACTGCCAGAGGCAGACCACCACTG 1632
       T  Y  S  S  L  H  R  G  N  C  Q  R  Q  T  T  T  V  530

DNA: TGATGGTGCCTGCTCACAAAGCTCAGCAGGAAGTGCTGCGTCTGCGCTACG 1683
       M  V  P  A  H  K  A  Q  Q  E  V  L  R  L  R  Y  D  547

DNA: ATGACTATGCCCAGTGTGTCTCAGACGATCATCTGATCAGGGTGAAAGCAT 1734
       D  Y  A  Q  C  V  S  D  D  H  L  I  R  V  K  A  F  564

DNA: TTGTAGAGGCTCCAGGCGAGAACGAGCCCCTCCTGACTGTGACTGACATCC 1785
       V  E  A  P  G  E  N  E  P  L  L  T  V  T  D  I  P  581

DNA: CACTCAGCAGACCTGAAGTCTTCATACAGGTTCCTGGGAGGGCTTTTGTTT 1836
       L  S  R  P  E  V  F  I  Q  V  P  G  R  A  F  V  C  598

DNA: GTGAACAAGTAAAAGCTTCCATCTCCTTCACCAATCCGTTACCAGTTCCAC 1887
       E  Q  V  K  A  S  I  S  F  T  N  P  L  P  V  P  L  615

DNA: TGAAAGGAGGTGTGTTCACTCTGGAGGGTGCAGGCCTACTCTCTTCCACTA 1938
       K  G  G  V  F  T  L  E  G  A  G  L  L  S  S  T  K  632

DNA: AGATCCATGTTAGTGGGGACATTTCTCCAGGACAGACGGTGTCCATTGTGG 1989
       I  H  V  S  G  D  I  S  P  G  Q  T  V  S  I  V  V  649

DNA: TGTCCTTCTCACCCATGAGGACTGGGGTGAGGAAGCTGCTGGTGGACTTTG 2040
       S  F  S  P  M  R  T  G  V  R  K  L  L  V  D  F  D  666

DNA: ACTCTGACCGACTGAAAGATGTAAAGGGAGTCACCACTGTGGTCGTCCGCA 2091
       S  D  R  L  K  D  V  K  G  V  T  T  V  V  V  R  K  683

DNA: AGAAGTACAGAAATATCTTCCCTGAAATTACTGAATGTTATTAAGTAACCT 2142
       K  Y  R  N  I  F  P  E  I  T  E  C  Y  *           696

DNA: TTAAATAATCTGAGACAATGTGACGTGTCCTGTGAGCGGATGAGCAATCGA 2193

DNA: GCAATCAGCATGTATGTACAATAAAACATGTTCCACATACATCAGCCTGTG 2244

DNA: AATGTCCACAAATACATCATGATAAAGCATTAGTTTGAAGCCACTATGTGT 2295

DNA: CAGGTATGAACATTTCTGACTTTGCATTATTATATTTTGCATTTTGTTTCG 2346

DNA: GTTTCACTGCGTTAGCGTCTCATTTCTTACCTTTGACTTTTGTTGTTTGGT 2397

DNA: TTTTCTGCTGAATTTTGGCGCCTGGCCGACAGTTTAGAGGTGAAAACGGTT 2448

DNA: TCTAATTAAAGAAATTCTGTTTTTGTTTGTAAAAAAAAAAAAAAAAAA    2493
```

Or the longer clone

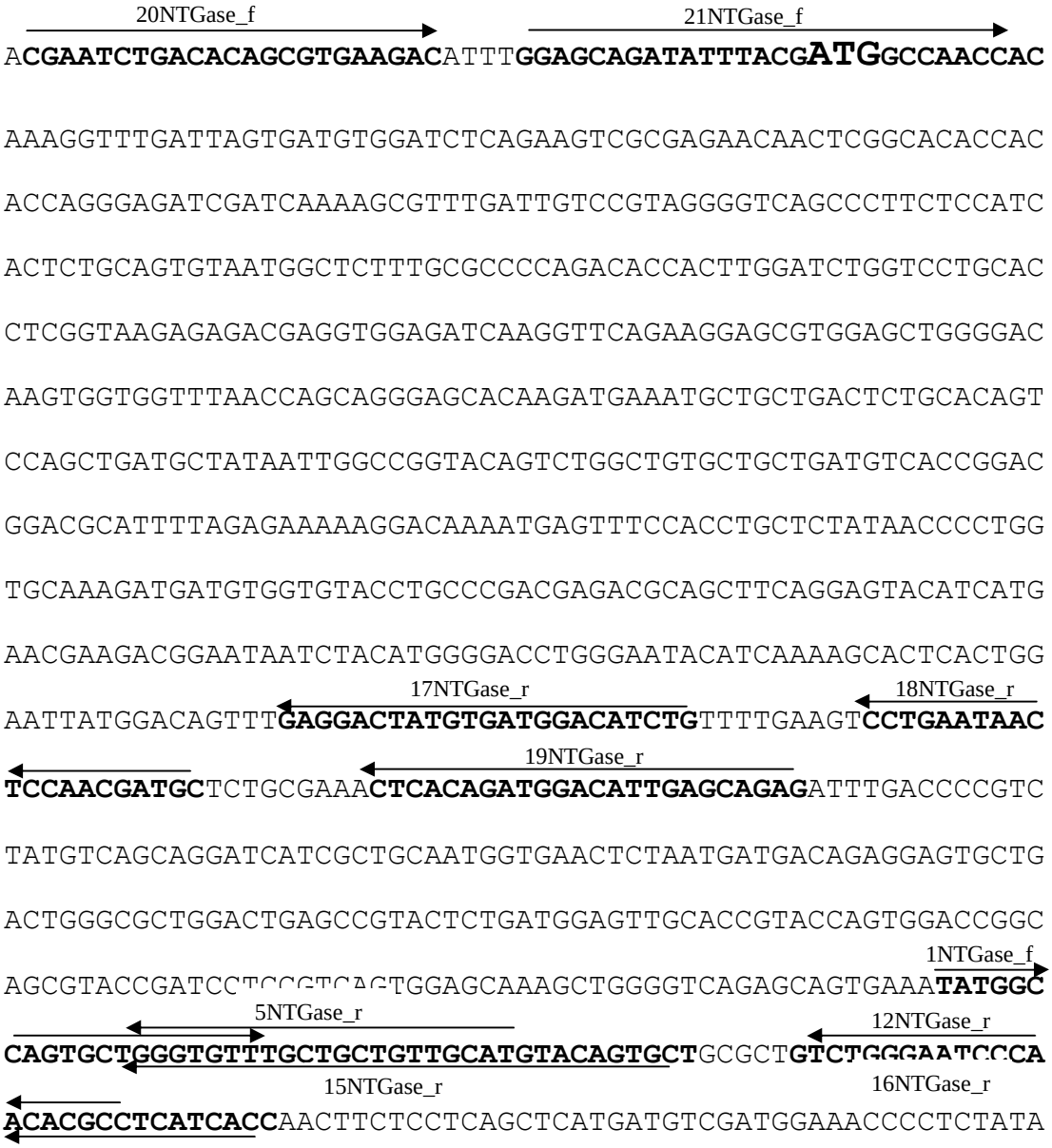
```
DNA: ....  similar to previous....  TTTTTTGTGTTTGTTTTTCA 2512

DNA: CTGTTTGTAATCACAAAGTTCAAATGAATGCACTTTTATAATATTTGTGTT 2563

DNA: TTAGCCAATATATAAAAACATATACAGAAAAAAAAAAAAAAAAAA      2594
```

Figure 22 The nucleotide sequence of Nile Tilapia TGase cDNA and deduce amino acid sequence. Nucleotide residues and amino acid residues are numbered from 5' end and the initiator methionine, respectively. The solid triangle indicates the Cys 272, His 332 and Asp 355 residues comprising the catalytic triad

.



GACTTCCTGTTGGATGAAAACCTGCAACCATTGGAGAGCAGAGGAGGGAGAGACAGC

9NTGase_f, 10NTGase_r

AGC**TGGAATTTCCACTGCTGGGTGGAG**TCTTGGATGAGGAGAGATGATCTCCCCAAA

7NTGase_f

GGAAATGATGGCTGGCAGGTTTTA**GATCCCACCCCTCAAGAA**CTCAGCAATGGGGTT

8NTGase_f

TTCCGCTGTGGTCCGTGCCCTGTGACAGCCATCAAGGAGGGAAATCTG**GGAGTGAAG**

4NTGase_f, 4NTGase_r

TACGATGCTCCG**TTTGTGTTTGCAGAGGTGAACGCTGA**CATCATCCACTGGATCGTT

CAGAGAAATGGCCAACGGAGAAAAATCAGAGTGGACCATGCCACTGTGGGTAGGAAC

ATCAGCACCAAAAGTGTTTATGGGGACTTCAGAGATGATGTCACTCTGCACTACAAA

TATCCTGAAGGATCCAAGAAGGAGAGAGAAGTGTACGAGAAGGCGGGGCGTCGGGTG

ACAGATGTCCCCAGTGAGAGCTCAGAACCAGGACGACTGCAGCTGTCCATCAAGCAT

GCCAAGCCTGTGTTTGGGACAGACTTTGATGTGATTATTGAGGTGAAGAATGAAGGA

GACCAGGATGCTCATGTTCAGCTGACCATGCTGGCTATGGCTGTTACTTACAGCTCT

CTTCATCGGGGGAACTGCCAGAGGCAGACCACCACTGTGATGGTGCCTGCTCACAAA

GCTCAGCAGGAAGTGCTGCGTCTGCGCTACGATGACTATGCCCAGTGTGTCTCAGAC

GATCATCTGATCAGGGTGAAAGCATTTGTAGAGGCTCCAGGCGAGAACGAGCCCCTC

CTGACTGTGACTGACATCCCACTCAGCAGACCTGAAGTCTTCATACAGGTTCCTGGG

AGGGCTTTTGTTTGTGAACAAGTAAAAGCTTCCATCTCCTTCACCAATCCGTTACCA

GTTCCACTGAAAGGAGGTGTGTTCACTCTGGAGGGTGCAGGCCTACTCTCTTCCACT

AAGATCCATGTTAGTGGGGACATTTCTCCAGGACAGACGGTGTCCATTGTGGTGTCC

TTCTCACCCATGAGGACTGGGGTGAGGAAGCTGCTGGTGGACTTTGACTCTGACCGA

CTGAAAGATGTAAAGGGAGTCACCACTGTGGTCGTCCGCAAGAAGTACAGAAATATC

TTCCCTGAAATTACTGAATGTTAT**TAA**GTAACCTTTAAATAATCTGAGACAATGTG

13NTGase_r

ACGTGTCCTGT**GAGCGGATGAGCAATCGAGC**AATCAGCATGTATGTACAATAAAACA

TGTTCCACATACATCAGCCTGTGAATGTCCACAAATACATCATGATAAAGCATTAGT

TTGAAGCCACTATGTGTCAGGTATGAACATTTCTGACTTTGCATTATTATATTTTGC

14NTGase_r

ATTTTGTTT**CGGTTTCACTGCGTTAGCG**TCTCATTTCTTACCTTTGACTTTTGTTGT

TTGGTTTTTCTGCTGAATTTTGGCGCCTGGCCGACAGTTTAGAGGTGAAAACGGTTT

CTAATTAAAGAAATTCTGTTTTTGTTTGTAAAAAAAAAAAAAAAAAA

Figure 23 All primers used in this study. The start and stop codon are highlight

```
Tilapia         MANHKGLISDVDLRSRENNSAHHTREIDQKRLIVRRGQPFSITLQCNGSLRPRHHLDLVL 60
red             MASYKGLIVDVNGRSHENNLAHRTREIDRERLIVRRGQPFSITLQCSDSLPPKHHLELVL 60
                **.:**** **: **:*** **:*****::****************..** *:***:***

Tilapia         HLGKRDEVEIKVQKERGAGDKWWFNQQGAQDEMLLTLHSPADAIIGRYSLAVLLMSPDGR 120
red             HLGKRDEVVIKVQKEHGARDKWWFNQQGAQDEILLTLHSPANAVIGHYRLAVLVMSPDGH 120
                ******** ******:** ************:********:*:**:* ****:*****:

Tilapia         ILEKKDKMSFHLLYNPWCKDDVVYLPDETQLQEYIMNEDGIIYMGTWEYIKSTHWNYGQF 180
red             IVERADKISFHMLFNPWCRDDMVYLPDESKLQEYVMNEDGVIYMGTWDYIRSIPWNYGQF 180
                *:*: **:***:*:*****:**:******::****:*****:******:**:*  ******

Tilapia         EDYVMDICFEVLNNSNDALRNSQMDIEQRFDPVYVSRIIAAMVNSNDDRGVLTGRWTEPY 240
red             EDYVMDICFEVLDNSPAALKNSEMDIEHRSDPVYVGRTITAMVNSNGDRGVLTGRWEEPY 240
                ***********:**  **:**:****:* *****.* *:******.********* ***

Tilapia         SDGVAPYQWTGSVPILRQWSKAGVRAVKYGQCWVFAAVACTVLRCLGIPTRLITNFSSAH 300
red             TDGVAPYRWTGSVPILQQWSKAGVRPVKYGQCWVFAAVACTVLRCLGIPTRPITNFASAH 300
                :******:********:********.************************ ****:***

Tilapia         DVDGNPSIDFLLDENLQPLESRGGRDSSWNFHCWVESWMRRDDLPKGNDGWQVLDPTPQE 360
red             DVDGNLSVDFLLNERLESLDSRQRSDSSWNFHCWVESWMSREDLPEGNDGWQVLDPTPQE 360
                ***** *:****:*.*:.*:**   ************** *:***:**************

Tilapia         LSNGVFRCGPCPVTAIKEGNLGVKYDAPFVFAEVNADIIHWIVQRNGQRRKIRVDHATVG 420
red             LSDGEFCCGPCPVAAIKEGNLGVKYDAPFVFAEVNADTIYWIVQKDGQRRKITEDHASVG 420
                **:* * ******:********************** *:****::******  ***:**

Tilapia         RNISTKSVYGDFRDDVTLHYKYPEGSKKEREVYEKAGRRVTDVPSESSEPGRLQLSIKHA 480
red             KNISTKSVYGNHREDVTLHYKYPEGSQKEREVYKKAGRRVTEPSNEIAEQGRLQLSIKHA 480
                :*********:.*:************:******:*******: ..* :* **********

Tilapia         KPVFGTDFDVIIEVKNEGDQDAHVQLTMLAMAVTYSSLHRGNCQRQTTTVMVPAHKAQQE 540
red             QPVFGTDFDVIVEVKNEGGRDAHAQLTMLAMAVTYNSLRRGECQRKTISVTVPAHKAHKE 540
                :**********:******.:***.***********.**:**:***:* :* ******::*


Tilapia         VLRLRYDDYAQCVSDDHLIRVKAFVEAPGENEPLLTVTDIPLSRPEVFIQVPGRAFVCEQ 600
red             VMRLHYDDYVRCVSEHHLIRVKALLDAPGENGPIMTVANIPLSTPELLVQVPGKAVVWEP 600
                *:**:****.:***:.*******:::***** *::**::**** **:::****:*.* *

Tilapia         VKASISFTNPLPVPLKGGVFTLEGAGLLSSTKIHVSGDISPGQTVSIVVSFSPMRTGVRK 660
red             LTAYVSFTNPLPVPLKGGVFTLEGAGLLSATQIHVNGAVAPSGKVSVKLSFSPMRTGVRK 660
                :.* :************************:*:***.* ::*. .**: :***********

Tilapia         LLVDFDSDRLKDVKGVTTVVVRKKYRNIFPEITECY 696
red             LLVDFDSDRLKDVKGVTTVVVHKKYRSLITGLHTD- 695
                *********************:****.:::.
```

Figure 24 Amino acid sequence alignment of Nile Tilapia liver TGase (this work)

and red sea bream liver TGase (AAB35370). The solid triangle indicates the Cys, His and Asp residues comprising the catalytic triad. The open box indicates the calcium binding site.

4.7 COMPARISON NILE TILAPIA TGase WITH OTHER TGase

The amino acid sequence of Nile Tilapia liver TGase showed 78%, 59%, 59% and 41% identity with TGase from red sea bream, chum salmon, zebra fish and chicken, respectively. The amino acid sequence of Nile Tilapia TGase aligned with various TGase are shown in figure 25.

```
CLUSTAL W (1.83) multiple sequence alignment

Nile            MANHK-----------------------------GLISDVDLRSRENNSAHHTREIDQK 30
red             MASYK-----------------------------GLIVDVNGRSHENNLAHRTREIDRE 30
chum            MTDQN-----------------------------GVFMGMDLLCQVNSHAHRTEEMDVE 30
zebrafish       MAPQVSAGSPNAGSWRECRPSGASGGKRVQCQWLSSILSDVDLQCYENNHAHRTEEMDVE 60
chicken         MAEEL-------------------------------VLETCDLQCERNGREHRTAEMGSQ 29
                *:                                   ::   :  .  *.  *:* *:. :

Nile            RLIVRRGQPFSITLQCNGSLR--PRHHLDLVLHLG------KRDEVEIKVQKERGAGDKW 82
red             RLIVRRGQPFSITLQCSDSLP--PKHHLELVLHLG------KRDEVVIKVQKEHGARDKW 82
chum            RLLVRRGQPFSLALQCHTTLP--PKHKLAIILHLG------KEGEVVVKVLDARAGRDKW 82
zebrafish       RLLVRRGQPFSVVLQCTEQIPQLPDHQINLILHLG------KNNEVVLKVSDSEQDPGKW 114
chicken         QLVVRRGQPFTITLNFAGRGYEEGVDKLAFDVETGPCPVETSGTRSHFTLTDCPEEGTWS 89
                :*:*******::.*:          .:: : :. *      .  .  ..: .

Nile            WFNQQGAQDEMLLTLHSPADAIIGRYSLAVLLMSPDGRILEKKDKMSFHLLYNPWCKDDV 142
red             WFNQQGAQDEILLTLHSPANAVIGHYRLAVLVMSPDGHIVERADKISFHMLFNPWCRDDM 142
chum            WFRQQGAQSEVLLTIHSPADAPVGLYSVTVLLLSPDGHILEKTTPETFYLLFNPWCKADS 142
zebrafish       WFSQRNAQGEVMLTLHSPADAPVGLYSMTVVLLSADGEIQEQTSPQTFYLLFNPWCKDDC 174
chicken         AVLQQQDGATLCVSLCSPSSARVGRYRLT--LEASTGYQGSSFHLGDFVLLFNAWHPEDA 147
                 . *:     : ::: **:.* :* * ::  : :. *   .      * :*:*.*   *

Nile            VYLPDETQLQEYIMNEDGIIYMGTWEYIKSTHWNYGQFEDYVMDICFEVLNNSNDALRNS 202
red             VYLPDESKLQEYVMNEDGVIYMGTWDYIRSIPWNYGQFEDYVMDICFEVLDNSPAALKNS 202
chum            VYLPDEELLEEYILNENGLLYQGSWDQISSLPWNFGQFEQDVVDICFEILDNSPAALTNP 202
zebrafish       VYLPSEEMLQEYILNENGILYQGAWDDITTVPWNFGQFEKDVVDICFDVLDNSPAALKNS 234
chicken         VYLKEEDERREYVLSQQGLIYMGSRDYITSTPWNFGQFEDEILAICLEMLDINPKFLRDQ 207
                *** .*   .**::.::*::* *: : * :  **:****. :: **:::*: .   * :

Nile            QMDIEQRFDPVYVSRIIAAMVNSND-DRGVLTGRWTEPYSDGVAPYQWTGSVPILRQWSK 261
red             EMDIEHRSDPVYVGRTITAMVNSNG-DRGVLTGRWEEPYTDGVAPYRWTGSVPILQQWSK 261
chum            EMDTANRADPVYVSRTITAMVNAND-DLGVVSGRWDGKYDDGVPPTRWTGSVPILRRWSE 261
zebrafish       EMDIFNRASPVYVSRTITAMVNAND-DRGVVSGRWDGEYSDGVAPTRWTGSVPILRRWSE 293
chicken         NLDCSRRNDPVYIGRVVSAMVNCNDEDHGVLLGRWDNHYEDGMSPMAWIGSVDILKRWRR 267
                ::*  .* .***:.* ::****.*. * **: ***   * **:.*  * *** **::* .
                      ▼                              ▽
Nile            AGVRAVKYGQCWVFAAVACTVLRCLGIPTRLITNFSSAHDVDGNPSIDFLLDENLQPLES 321
red             AGVRPVKYGQCWVFAAVACTVLRCLGIPTRPITNFASAHDVDGNLSVDFLLNERLESLDS 321
chum            AGAQRVRYGQCWVFSGVACTVLRCLGIPTRPVTNYSSAHDTDGNLNVDYLYDEQLESVSE 321
zebrafish       DGGQKVRYGQCWVFTGVACTVLRCLGIPTRCITNYSSAHDTDANIAVDYLVNDQLESVSE 353
chicken         LGCQPVKYGQCWVFAAVACTVMRCLGVPSRVVTNYNSAHDTNGNLVIDRYLSET--GMEE 325
                 * : *:*******:.*****:****:*:* :**: ****.:.*  :*   .:    :..
```

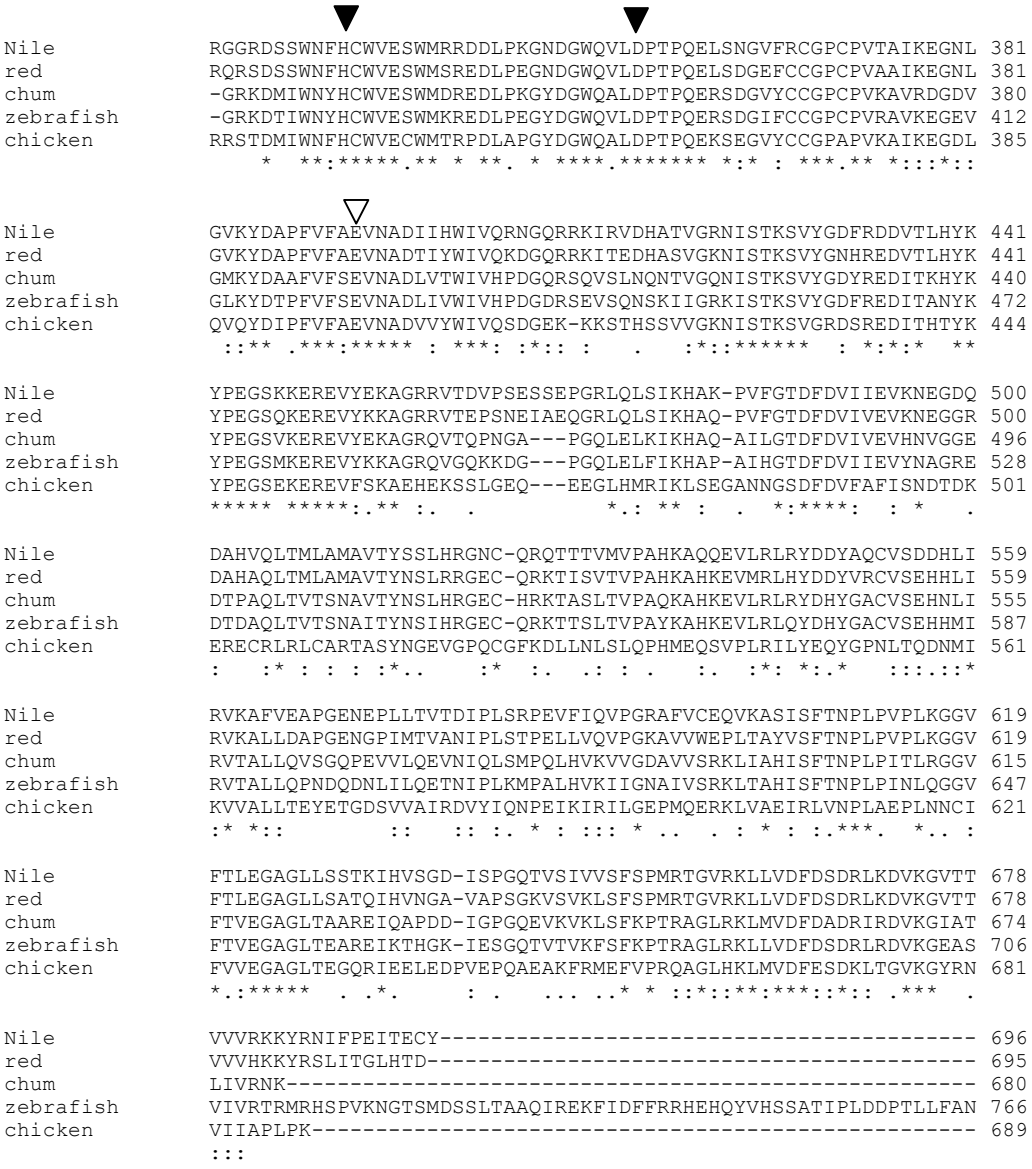


Figure 25 Multiple sequence alignment of TGase from Nile Tilapia (this work, DQ480532), red sea bream (AAB35370), chum salmon (BAA11633), zebra fish (XP_690450) and chicken (NP_990779). The solid triangle indicates the catalytic triad. The open triangles indicates the His and Glu residues that are critical for catalytic activity.

CHAPTER V

CONCLUSIONS

1. Four primers for partial cDNA synthesis were designed from nucleotide sequence alignment of TGase from three organisms, red sea bream, salmon and the 2 groups of Nile Tilapia muscle sequences.

2. Partial cDNA amplification was amplified with primer 1NTGase_f and 11NTGase_r. Then semi-nested PCR was done by using primer 1NTGase_f and 4NTGase_r. From the partial cDNA sequencing result, primers for 3' and 5' end cDNA were designed.

3. 3' RACE amplification was done by 2 steps RT-PCR with primers 8NTGase_f and Qi. The PCR products showed double band, from the sequencing result both of them were TGase gene with differences in 3' UTR that concern to polyadenylation site.

4. 5' RACE amplification was done twice. First time, the first strand cDNA was synthesized with primer 10NTGase_r. PCR amplification was done with primer 16NTGase_r and AAP. The sequencing result showed 68% identity to red sea bream TGase but did not reach the 5' cDNA end. Second time, first strand cDNA was synthesized with primer 19NTGase_r. PCR amplification was done with primer 17NTGase_r and AAP. The sequencing result showed 78 % identity to red sea bream TGase and reach to 5' UTR sequence.

5. Full length cDNA was amplified with primers 20NTGase_f and 14NTGase_r. From the sequencing result, full length cDNA sequence consists of 2,493 nucleotides with an open reading frame of 2,091 nucleotides coding for 696 amino acids. The amino acid sequence of Nile Tilapia liver TGase showed 78%, 59%, 59% and 41% with TGase from red sea bream, chum salmon, zebra fish and chicken, respectively. The catalytic triad of Nile Tilapia consists of Cys 272, His 332 and Asp 355. The Nile Tilapia TGase had an extension of 1 amino acid in the C-terminal region and some differences in the N-terminal region when compared with red sea bream TGase. The calculated molecular weight of Nile Tilapia TGase was 78.9 kDa and with an isoelectric point of 6.31.

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APPENDIX

APPENDIX I

1. 1 MEDIA

1.1.1 LB medium (Luria-Bertani Medium) Per liter:

deionized H_2O	950 ml
tryptone	10 g
yeast extracts	5 g
NaCl	10 g

Stir until the solutes have been dissolved. Adjust the pH to 7.0 with 5 N NaOH. Adjust the volume of the solution to 1 liter with deionized H_2O. Sterilized by autoclaving for 20 minutes at 15 psi on liquid cycle.

1.1.2 LB agar Per liter:

deionized H_2O	950 ml
tryptone	10 g
yeast extracts	5 g
NaCl	10 g
agar	15 g

Stir until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH. Adjust the volume of the solution to 1 liter with deionized H_2O. Sterilize by autoclaving for 20 minutes at 15 psi on liquid cycle.

1.1.3 SOC per liter:

deionized H_2O	950 ml
tryptone	20 g
Yeast extracts	5 g
NaCl	0.5 g

After media has been autoclaved, allow it to cool to 60 °C or less. Add 20 ml of sterile 1M glucose solution to the media.

1.2 REAGENT

1.2.1 10x Tris EDTA (TE) pH 8.0

100 mM Tris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Sterilize solution by autoclaving for 20 minutes at 15 psi on liquid cycle. Store the buffer at room temperature.

1.2.2 Tris-Cl

Dissolved 121.1 g of Tris base in 800 ml of H_2O. Adjust the pH to the desired value by adding concentrated HCl

pH	HCl
7.4	70 ml
7.5	60 ml
8.0	42 ml

Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 liter with H_2O. Dispense into aliquots and sterilize by autoclaving.

If 1 M solution has a yellow color, discard it and obtain Tris of better quality. The pH of Tris solution is temperature-dependent and decrease ~ 0.03 pH units for each 1 °C increase in temperature. For example, a 0.05 M solution has pH values of 9.5, 8.9 and 8.6 at 5 °C, 25°C and 37 °C respectively.

1.2.3 Extraction / Lysis Buffer and Solution for plasmid extraction

1.2.3.1 Alkaline lysis Solution I (plasmid preparation)

50 mM glucose

25 mM Tris-Cl (PH 8.0)

10 mM EDTA

Prepare solution I from standard stock in batches of ~ 100 ml, autoclave for 15 minutes at 15 psi on liquid cycle, and store at 4 °C

1.2.3.2 Alkaline lysis Solution II (plasmid preparation)

0.4 N NaOH

2% SDS

Prepare solution II fresh and use at room temperature

1.2.3.3 Alkaline lysis Solution III (plasmid preparation)

5 M Potassium acetate	60.0 ml
glacial acetic acid	11.5 ml

H_2O	28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store the solution at 4 °C and transfer it to an ice bucket before use.

1.2.4 Electrophoresis and gel loading buffer

1.2.4.1 TAE buffer 50X

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

1.2.4.2 Gel loading buffer

bromophenol blue	0.25 % (w/v)
xylene cyanol FF	0.25 % (w/v)
glycerol in H_2O	30 % (v/v)

Store at 4 °C

1.2.5 Chemical stock solution

1.2.5.1 Glycerol (40% v/v)

Dilute 40 ml of molecular biology grade glycerol in 60 ml of sterilized pure H_2O. Sterilize the solution by passing it through a prerinsed 0.22 μM filter, store at 4 °C

1.2.5.2 IPTG (20% w/v, 0.8 M)

IPTG is isopropylthio-β-D-galactoside. Make a 20 % solution of IPTG by dissolving 2 g of IPTG in 8 ml of distilled H_2O. Adjust the volume of the solution to 10 ml with H_2O and sterilize by passing it through a prerinsed 0.22 μM filter. Dispend the solution into 1 ml aliquots and store them at -20 °C

1.2.5.3 X-gal Solution (2% w/v)

X-gal is 5- bromo-4-chloro-3-indolyl- β-D-galactoside. Make a stock solution by dissolving X-gal in dimethylformamide at concentration of 20 mg/ml solution. Use a glass or polypropylene tube. Wrap the solution in aluminum foil to prevent damage by light and store at -20 °C. It is not necessary to sterilize X-gal solution by filtration.

1.3 STORAGE BACTERIA CULTURE

Storage of bacteria culture growing in liquid media

1. To 1.5 ml of bacteria culture, add 0.5 ml of sterile 60 % glycerol (sterilized by autoclaving for 20 minutes at 15 psi on liquid cycle)

2. Vortex the culture to ensure that the glycerol is evenly dispersed.

3. Transfer the culture to labeled storage tube equipped with a screw cap and an air-tight gasket.

4. Freeze the culture in liquid nitrogen, and then transfer the tube to - 70°C for long term storage.

5. To recover the bacteria, scrape the frozen surface of the culture with sterile

inoculating loop, and then immediately streak the bacteria that adhere to the loop onto the surface of the LB agar plate containing the appropriate antibiotic. Return the frozen culture to storage at - 70°C. Incubate the plate overnight at 37 °C.

APPENDIX II

2.1 PROTOCOL FOR LIGATION USING THE pGEM-T EASY VECTOR

2.1.1 Set up the ligation reactions as describe below

2X rapid ligation buffer	5 µl
pGEM-T easy vector	1 µl
PCR product	3 µl
T4 DNA ligase	1 µl

2.1.2 Mix the reactions by pipetting. Incubate the reactions overnight at 4 °C.

Note:

- Use only Promega T4 DNA ligase supplied with the system in performing pGEM-T easy vector ligation. Other commercial preparation of T4 ligase may contain exonuclease activities that move the terminal deoxythymidines from the vector.

- 2X rapid ligation buffer contain ATP, which degrade during temperature fluctuations. Avoid multiple freeze-thaw cycles and exposure to frequent temperature change by making single-use aliquots of buffer.

- It is important to vortex the 2X rapid ligation buffer before each use.

- Incubate the reactions overnight at 4 °C will produce the maximum number of transformation

2.2 PROTOCOL FOR TRANSFORMATION USING THE pGEM-T EASY VECTOR LIGATION REACTION

2.2.1 Prepare 2 LB/ampicillin/IPTG/X-Gal plate for each ligatrion reaction, equilibrate the plat at 37 ℃, 30 minutes prior to plating.

2.2.2 Centrifuge the tube containing the ligation reaction to collect contents at the bottom of the tube. Add 5 µl of ligation reaction to a sterile 1.5 ml micro-centrifuge tube on ice.

2.2.3 Remove tube of frozen Top10 competent cell from -70 ℃ storage and place in the ice bath until just thawed.

2.2.4 Carefully transfer competent cell into the tube prepared in step 2.

2.2.5 Gently mix and place them on ice for 20 minutes.

2.2.6 Heat-shock the cell for 45-50 seconds in heat box at exactly 42 ℃ (do not shake).

2.2.7 Immediately return the tube to ice for 2 minutes.

2.2.8 Add 250 room temperature SOC medium to the tube containing cells transformed with ligation reaction.

2.2.9 Incubate at 37 ℃ for 1 hour with shaking (~150 rpm).

2.2.10 Plate 150 µl of the transformation culture onto duplicate LB/ampicillin/ IPTG/X-Gal plate.

2.2.11 Incubate plate overnight at 37 ℃.

2.3 PLASMID PREPARATION BY USING QIA PREP MINIPREP

2.3.1 Resuspend pellet bacteria cell in 250 µl of Buffer P1 then vortex.

2.3.2 Add 250 µl of Buffer P2 and gently invert tube 4-6 times to mix (do not vortex).

Do not allow the lysis reaction to proceed for more than 5 minutes.

2.3.3 Add 350 µl of Buffer N3 and invert the tube immediately but gently 4-6 times.

2.3.4 Centrifuge for 10 minutes.

2.3.5 Apply the supernatants from step 4 to the QIAprep column by pipetting.

2.3.6 Centrifuging 30-60 seconds, discard the flow-through.

2.3.7 (Optional): Wash QIAprep spin column by adding 0. 5 ml of Buffer PB and centrifuging 30-60 seconds, discard the flow-through.

2.3.8 Wash QIAprep spin column by adding 0.75 ml of Buffer PE and centrifuging 30-60 seconds.

2.3.9 Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.

2.3.10 Place QIAprep column in a clean 1.5 ml microfuge tube. To elute DNA, add 50 µl of Buffer EB (10mM Tris-cl, pH8.5) or H_2O to the center of each QIAprep column, let stand for 1 minute, and centrifuge for 1 minute.

2.4 DNA EXTRACTION FROM AGAROSE FEL

2.4.1 Excise DNA fragment; Take a clean scalpel to excise the DNA fragment from agarose gel. Excise gel slice containing the fragment carefully to minimize the gel volume. Determine the weight of the gel slice and transfetr it to a clean tube.

2.4.2 Gel lysis; For each 100 mg agarose gel add 200 µl NT buffer. Incubate at 50 °C until the gel slices are disslloved (5-10 minute). Vortexes the samples briefly every 2-3 minute until the gel slice are dissolved completely.

2.4.3 Bind DNA; Place a NucleoSpin Extract II column into a 2 ml collecting

tube and load the sample. Centrifuge for 1 min at 11,000 x g. Discard flow-through and place the NucleoSpin Extract II column back into the collecting tube.

2.4.4 Wash silica membrane; Add 600 µl buffer NT3. Centrifuge for 1 minute at 11,000 x g. Discard the Discard flow-through and place the NucleoSpin Extract II column back into the collecting tube.

2.4.5 Dry silica membrane; Centrifuge for 2 minute at 11,000 x g to remove buffer NT3 quantitatively. Make sure the spin column doesn't come in contact with the flow-through while removing it from the centrifuge and the collecting tube.

2.4.6 Elute DNA; Place the NucleoSpin Extract II column into a clean 1.5 ml microcentrifuge tube. Add 15-50 µl elution buffer NE, incubate at room temperature for 1 minute to increase the yield of eluted DNA. Centrifuge for 1 minute at 11,000 x g.

2.5 S.N.A.P column purification of cDNA

2.5.1 Equilibrate the binding solution to room temperature.

2.5.2 For each sample to be purified, equilibrate ~ 100 µl of sterilized, distilled water at 65 °C for use in step 9.

2.5.3 Add 120 µl of binding solution (6M NaI) to the first reaction.

2.5.4 Transfer the cDNA/NaI solution to a S.N.A.P column. Centrifuge at 13,000 x g for 20 seconds.

2.5.5 Remove the cartridge insert from the tube and transfer the flow-through to a microcentrifuge tube. Save the solution until recovery of the cDNA is confirmed. Place the cartridge insert back into the tube.

2.5.6 Add 0.4 ml of cold (4 °C) 1X wash buffer to the spin cartridge. Centrifuge at 13,000 x g for 20 seconds. Discard the flow-through. Repeat this wash step three

additional times.

2.5.7 Wash the cartridge two times with 400 µl of cold (4 °C) 70 % ethanol as described in step 6.

2.5.8 After removing the final 70 % ethanol wash from the tube, centrifuge at 13,000 x g for 1 minute.

2.5.9 Transfer the spin cartridge insert into a fresh sample recovery tube. Add 50 µl of sterilized, distilled, water (preheat to 65 °C) to the spin cartridge. Centrifuge at 13,000 x g for 20 seconds to elute the cDNA.

BIBLIOGRAPHY

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