Highlights

- Transglutaminases catalyze the crosslink formation between glutamine and lysine residues
- TG2, among transglutaminase family, contribute to a critical role in tissue fibrosis
- We introduce the method for measuring isozyme-specific transglutaminase activity
- We recently developed methods for detection and identification of transglutaminase substrates.
- We evaluated the specificity and similarity of possible transglutaminase substrates identified

Identification and characterization of substrates crosslinked by transglutaminases in liver and kidney fibrosis

Hideki Tatsukawa*, Taishu Takeuchi, Yoshiki Shinoda, Kiyotaka Hitomi

Department of Basic Medicinal Sciences, Graduate School of Pharmaceutical Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan

*Correspondence: *Hideki Tatsukawa, PhD.* Tel. +81-52-747-6808; Fax +81-52-747-6810; E-mail address: htatsukawa@ps.nagoya-u.ac.jp

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Running title: Characterization of TGase substrates in tissue fibrosis

Abbreviations used in this article

BDL Bile duct ligation
BPA Biotinylated pentylamine
ECM Extracellular matrix
FXIII Factor XIII
SDS-PAGE Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
TGase Transglutaminase
TGF-β Transforming growth factor-β
UUO Unilateral ureteral obstruction

ABSTRACT

Transglutaminases (TGase) family consists of eight isozymes, which catalyze the Ca^{2+}-dependent crosslink formation between glutamine and lysine residues of proteins. In the pathogenesis of a wide variety of chronic disease, especially TG2 among the TGase isozyme was upregulated and contribute to a critical role in fibrosis development and progression via stabilization of extracellular matrix proteins and the activation of TGF-β. Although TG2 has been implicated as a key enzyme in fibrosis, the causative role of TG2 and involvement of other isozymes remains unelucidated. We recently developed a comprehensive analysis method targeting isozyme-specific substrates of TGase in liver and kidney fibrosis. In this study, we introduce the previously developed method for the measuring activity and tissue distribution of TGase and for detection and identification of TGase substrates in an isozyme-specific manner. Using our comprehensive analysis method, we characterized the possible substrates of TG1 and TG2 in fibrotic tissue from different organs. By comparing the specificity and similarity of possible TGase substrates between different models of tissue fibrosis, our results provide a deeper understanding about the specific and common pathways in disease pathogenesis and progression.

1. Introduction

Transglutaminases (TGase) is a family of multifunctional enzymes comprising eight isozymes, such as TG1–7 and blood coagulation factor XIII (FXIII), all of these enzymes share a common structure and primarily form. These enzymes are responsible for the formation of N^{ε}-(γ-glutamyl)-lysine crosslinking between glutamine and lysine residues in Ca^{2+}-dependent posttranslational modification [1]. TGases are widely distributed and each isozyme is involved in multiple biological processes (Table 1). TGases catalyze the deamidation of γ-carboxamide groups of a protein/polypeptide glutaminyl residue or convert glutamine to glutamic acid. While TGases catalyze both transamidation and deamidation reactions, the directional regulations and specific functional substrates remain unknown.

To understand the direct functional roles of TGase, identification and analysis of specific substrates in each physiological and pathological event are required. To date, several substrates for each TGase isozyme have been characterized using recombinant proteins or cellular models. However, these substrates, which were identified *in vitro,* cannot be applied to understanding of *in vivo* reactions. Based on these considerations, well-designed experiments to analyze TGase are required to demonstrate clear evidence that can be reflected in *in vivo* finding.

Several methods to identify the reactive residues of both glutamine and lysine have been developed. Initial experiments identified the crosslinking site by sequencing peptides [2]. Following the sequencing of peptides containing a ε-(γ-glutamyl) lysyl bridge, two NH_2-terminal amino acids of equimolar ratio were detected. Because the isopeptide bond is resistant to Edman degradation, this crosslinked pair of residues is released as bis-phenylthiohydantoin-ε-(γ-glutamyl) lysine. Since the development of mass spectrometry, new strategies based on functional proteomic analyses have utilized labeled acyl- and amine-donor probes as TGase substrates; *i.e.,* synthetic biotinylated peptide TVQQEL and biotinylated pentylamine (BPA) [3].

Since their identification, the interactions between TGases and their substrates have been investigated. TGases exhibit high substrate specificity and require certain amino acid sequence for catalysis. Cousson et al. have proposed the minimal requirements for TGase-catalyzed modification of a putative glutamine site in a substrate protein. The amino acid sequence around the glutamine should be accessible and in a highly flexible region. In addition, the amino acid sequence needs to allow proper interaction with TGase, which is mainly dependent on the charge of the residues on the C-terminal side of glutamine [4].

Moreover, these preferences regarding recognition sequence against the substrates are difference for each TGase isozymes. Previously, we screened the potential peptide sequences recognized by each TGase isozyme from 12-mer random peptide library using M13 phage display [5–10]. The screening for TG2 highlighted marked tendencies in glutamine-donors

sequences QXP□D(P), QXP□, and QXX□DP (where X and □ represent any amino acid and any hydrophobic amino acid, respectively). At the same time, Fesus' group also screened glutamine-donor substrates from 7-mer random peptide library by binding to GST-fused recombinant TG2 [11]. Although different screening approaches were used, their findings (pQX(P,T,S)l; where p and l stands for polar and aliphatic amino acids, respectively) were consistent with our results described above.

Moreover, Facchiano's group developed a database to assess the possible substrates by analyzing their amino acid sequences (TRANSIT, http://bioinformatica.isa.cnr.it/TRANSIT/) [12]. In addition, the TRANSDAB wiki (http://genomics.dote.hu/wiki/index.php/Main_Page), database that lists about 350 substrates for each TGase isozyme and their interaction partners, was developed by Fesus' group [13]. Because the last update of this database was in 2010, the number of substrates must have increased due to recent studies using genomic and proteomic approaches. Recently, Nikolajsen et al. reported the proteomic approach for global identification of FXIIIa substrates in plasma by labeling BPA [14]. In addition, Verderio's group developed a global proteomic approach to identify binding partners of TG2 responsible for TG2 externalization in kidney fibrosis [15]. Although they only focused on proteins binding with TG2, the identified proteins included the substrates of TG2, which bind to TG2 in the substrate recognition step.

Similar to these studies, we have recently developed a global identification method that involves targeting isozyme-specific substrates of TGase in cells [7,16] and tissues [17–19]. In this study, we introduce the global identification method we developed and the profile of possible substrates that have been identified so far. The information we provided could be widely adopted to several research fields related to TGase and help clarify the novel roles of TGases in both physiological and pathological events.

1.1. Characterization of the preferred substrate sequences recognized by each TGase

To date, we have established a novel screening procedure and identified the primary structure sequence that was specifically recognized by each TGase isozyme using a random peptide library. During the formation of the enzyme-substrate intermediate, the glutamine residue of the substrate is selected for interaction with the active cysteine residue of the TGase. Therefore, the feasibility of the interaction with amino acid sequences surrounding glutamine is quite important in deciding the speed and efficiency of the catalytic reactions. We have obtained the preferred substrate peptides for isozymes FXIII, TG1, TG2, TG3, TG6, and TG7 [5–10] (Table 1). In addition, we obtained the specific substrate sequence for microbial transglutaminase (MTG) from *Streptomyces mobaraensis* that has been used in the food industry [20]. Each peptide sequence, even in the primary structure, showed specificity and high reactivity for the reaction catalyzed by TGase. For the lysine-donor substrate, there appeared to be a preferred sequence, which was less specific compared with the glutamine-donor substrate. Currently, we have failed to obtain specific sequences for the lysine-donor substrate. We suppose that our screening conditions might not apply to analyzing sequences with a lower specificity.

1.2. Observation of the tissue distribution of TGase activity based on the detection of possible substrates

As mentioned above, preferred substrate peptides can be used in several assays by labeling them with fluorescent dyes or biotin. The labeled substrate peptides are incorporated into the lysine residues of the target substrate protein through TGase isozyme-specific reactions. The labeled primary amine, such as BPA, has also been used to measure TGase activity and detect substrates because primary amine is also incorporated into glutamine residue of substrates. However, this system using primary amine cannot be applied in an isozyme-specific manner. By taking advantage of the specificity and sensitivity of substrate peptides, we developed a system for *in situ* detection of TGase activity using fluorescently-labeled peptides, which enables visual localization of enzyme activity (Fig. 1A). On an unfixed frozen tissue section, the lysine-donor of substrates was crosslinked with each FITC-labeled substrate peptide and visualized as a fluorescent signal. The advantage of this method is that it can only detect the active form of each TGase isozyme, whereas immunostaining is unable to discriminate between the active and inactive forms of TGase. In fact, the distribution patterns determined from *in situ* activity and immunostaining in various tissues are often different, as shown in our previous studies [21,22]. Using this *in situ* detection system, we showed the different localization of each TGase isozyme activity in a liver and kidney fibrosis model induced by bile duct ligation (BDL) and unilateral ureteral obstruction (UUO), respectively (Fig. 2B). The isozyme-specific activities of TG1 and

TG2 were measured using the substrate peptides pepK5 and pepT26, respectively (Fig. 1B). Seven days after BDL surgery, the results indicated that TG1 activity enhanced significantly over a widespread area of the fibrotic liver section, whereas TG2 activity was limited to the periportal area in the control and the extracellular space in the fibrotic liver section. The combined pattern of TG1 and TG2 activities was observed by incorporating BPA as an amine donor (*data not shown*). Three days following UUO surgery in the kidney section, TG1 activity was enhanced in the renal tubule at the early fibrotic stage, whereas TG2 activity enhanced significantly in the interstitial area. In both the cases, no fluorescent signals were observed in reactions using mutant pepK5 and pepT26, in which glutamine is replaced with asparagine (*data not shown*).

1.3. Detection, purification, and identification of the substrate proteins in an isozyme-specific manner

The substrate peptide, including glutamine and primary amine, can be used for the detection, purification, and identification of the possible substrate proteins. The incubation of cellular soluble extracts with biotin-labeled peptides generates crosslinked products whose reactive glutamine residues incorporate the biotin-substrate peptides. By utilizing these biotin-labeled substrate peptides, we were able to detect the various possible substrates using a peroxidase-conjugated streptavidin on the membrane blotted after SDS-PAGE (Fig. 2A). Consistent with results from the *in situ* activity staining (Fig. 1), activities of both TG1 and TG2 were enhanced marginally, and several possible glutamine-acceptor substrates incorporating each peptide increased in the fibrotic livers in a time-dependent manner, following BDL surgery (Fig. 2B).

2. Characterization of identified TG1 and TG2 possible substrates in the liver and kidney fibrotic tissues

As shown in Fig. 2, pepK5 and pepT26 were incorporated into various proteins in the fibrotic tissue, indicating that these modified proteins may be possible substrates for TG1 and TG2, respectively. We next identified the proteins incorporated with these biotinylated substrate peptides as well as with BPA. Figure 3 shows a schematic diagram of the purification and identification of possible lysine- or glutamine-donor substrates. Each biotinylated substrate peptide was incubated with the tissue extracts, and the peptide-incorporated proteins were then purified using monoavidin resin and subjected to trypsin digestion. The fragmented peptides were fractionated by nano-HPLC and identified using a MALDI-TOF/ TOF mass spectrometer. In the liver fibrosis model induced by BDL surgery, we identified 43 and 42 of the possible

substrates for TG1 and TG2, respectively, that contained at least one reactive lysine residue only in the fibrotic liver and not in the untreated control liver (Fig. 4A; [18]). In addition, a total of 65 unique proteins incorporating BPA that contained at least one glutamine residue were also identified as TGase substrates (Fig. 4A; [18]). Only five proteins (fibrinogen gamma chain, keratin 18, myosin-1, myosin-9, and serotransferrin) were identified as both lysine-donor and glutamine-donor in the fibrotic liver. Although fibrinogen alpha was also reported as a crosslinking substrate with fibrinogen gamma [23], we did not identify this substrate, suggesting that the types of substrates identified might be due to protein solubility and ionization tendency. Similar studies were performed in the kidney fibrosis model induced by UUO surgery. This resulted in the identification of 47 and 67 possible substrates for TG1 and TG2, respectively, all of which were only identified in the fibrotic kidney and not in the untreated control (Fig. 4B; [17]). A total of 116 unique proteins incorporating BPA were also identified as TGase substrates. Interestingly, a large number and high frequency of proteins identified in the kidney fibrosis model as substrates of both lysine-donor and glutamine-donor (15 proteins; 9%) compared with the liver fibrosis model (5 proteins; 4%). These proteins were cytoplasmic actin 1, complement factor H, heterogeneous nuclear ribonucleoproteins A/B, F, H U, lactotransferrin, moesin, nuclease-sensitive element-binding protein 1, protein S100-A9, serotransferrin, talin-1, tubulin beta-2B/5 chain, and uromodulin. To characterize the possible substrates identified in the fibrotic liver and kidney, the overlapped proteins were graphed as shown in Fig. 4C. The rate of overlapped proteins that incorporated BPA (substrates for TGase; 10%) in both liver and kidney fibrosis model was higher than the rate of overlapped proteins that incorporated substrate peptides (substrates for TG1 or TG2; 6%). These overlapped substrates incorporating pepK5, pepT26, and BPA are individually listed in Tables 2–4. Serotransferrin/transferrin is the major plasma iron-transport protein in mammals and is involved in the development of fibrosis in several tissues. Serum transferrin has been identified as a biomarker for liver fibrosis in recent years [24,25]. As listed in Tables 2–4, serotransferrin was incorporated with pepK5, pepT26, and BPA in both liver and kidney fibrosis model, which might suggest that it could be a broad biomarker for fibrosis with no tissue-type specificity.

3. Concluding remarks

In this article, we introduced the availability of substrate peptides that recognize possible substrates of TGase in an isozyme-dependent manner and we reported applicability of these peptides for analyzing the activity and distribution of TGase. We also performed a comprehensive analysis for detection and identification of TGase substrates in the liver and kidney fibrosis models. Through the identification of substrates, we determined that the rate of

overlapping proteins that incorporated BPA were higher than those that incorporating our novel substrate peptides (pepK5 and pepT26). These results were reasonable because the isozyme-specificity of BPA is lower than that of substrate peptides. In our approach, analyzing the similarities of TGase substrates between several fibrotic tissues from different organs contributed to a deeper understanding of key pathways in disease pathogenesis and progression. Although further investigation of the unique substrates related to fibrosis development is ongoing, these finding might facilitate the development of diagnostic methods and new drugs for disease treatment and antifibrotic therapies.

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Fig. 1. Observation of TGase crosslinking activity and distribution on unfixed tissue section using substrate peptide

(A) Unfixed tissue section was incubated with each substrate peptide labeled with fluorescein and then the sections were washed and observed under a fluorescence microscope. (B) The fibrosis model in liver or kidney was produced by BDL (Day 7) and UUO (Day 3) surgery, respectively. The in situ activities of TG1 and TG2 were visualized using FITC-labeled substrate peptides (pepK5 and pepT26, respectively). Bar = 50 μ m.

Fig. 2. Detection of possible substrates incorporated with each substrate peptide in liver extracts

(A) Tissue extracts were incubated with each biotinylated substrate peptide, and resultant proteins that incorporated the substrate peptide were subjected to detection using peroxidase-conjugated streptavidin on the membrane blotted after SDS-PAGE. (B) The liver extract at each indicated day time point were incubated with either pepK5 or pepT26, and then the amount of Lys-donor substrates incorporated with each biotinylated peptide on the blotting membrane was detected using peroxidase-conjugated streptavidin. The sizes of the protein mass markers are shown on the left.

Fig. 3. Schematic diagram for purification and identification of possible lysine- or glutamine-donor substrates

Tissue extracts were incubated with each biotinylated substrate peptide (A; pepK5 and pepT26) and pentylamine (B; BPA), following the same procedure as outlined above for the in vitro detection of activity (Fig. 2). Following the crosslinking reaction by the endogenous TGase in the tissue extracts, these samples were applied to monoavidin resin for purification of crosslinking products. The samples eluted by excess biotin were precipitated with TCA/acetone, and then subjected to trypsin digestion for the identification of possible substrates using MALDI-TOF/TOF mass spectrometer after fractionation using a reverse-phase Nano-HPLC in a C18 column.

Fig. 4. A comparative proteomic analysis of possible substrates incorporating pepK5, pepT26, and BPA in liver and kidney fibrosis

From data in the previous our reports [17,18], the number and rate of identified proteins incorporating pepK5, pepT26, and BPA in each sample from liver fibrosis (A) and kidney fibrosis (B) were described as Venn diagram. The overlapped number and rate of identified proteins were also indicated. (C) Comparison analyses of overlapped proteins between liver (BDL) and kidney fibrosis (UUO) in pepK5, pepT26, and BPA-incorporated substrates were also indicated as Venn diagram. These each overlapped proteins are listed in Tables 2-4.

Table 1. Members of the mammalian transglutaminase (TGase) family, tissue distribution, biological functions, and amino acid sequence of our developed substrate peptide

TGase	Tissue distribution	Biological function	Sequence of substrate peptide
FXIII	Plasma, brain, bone	Blood clotting, bone growth	DQMMLPWPAVAL (pepF11)
TG1	Epithelia	Barrier function in epithelia	YEQHKLPSSWPF (pepK5)
TG2	Ubiquitous	Cell death, survival signal, cell adhesion, fibrosis, and so forth	HQSYVDPWMLDH (pepT26)
TG3	Epidermis, hair follicle	Terminal differentiation of keratinocytes, hair follicles	PPPYSFYQSRWV (pepE51)
TG4	Prostate gland	Semen coagulation (rodents)	Yet to be developed
TG5	Foreskin keratinocytes, female reproductive tissues, skeletal muscle	Epidermal differentiation	Yet to be developed
TG6	Epidermis, testis, brain	Formation of epidermis and hear follicle, neuronal development	DDWDAMDEQIWF (pepY25)
TG7	Testis, kidney	Unknown	YSLQLPVWNDWA (pepZ3S)

Table 2. The list of overlapped proteins between liver and kidney fibrosis in pepK5-incorporated substrates.

TG1 (pepK5)		Liver sample from each day after BDL (days)				Kidney sample from each day after UUO (days)			
Accession #	Name	0	3	7	14	0	3	7	14
Q8K0E8	Fibrinogen beta chain		+	+			+	+	+
Q8VCM7	Fibrinogen gamma chain		+					+	+
P01942	Hemoglobin subunit alpha		+						+
Q921I1	Serotransferrin				+		+	+	+
P68373	Tubulin alpha-1C chain		+	+	+		+	+	+
Q9CWF2	Tubulin beta-2B chain		+					+	

Table 3. The list of overlapped proteins between liver and kidney fibrosis in pepT26-incorporated substrates.

TG2 (pepT26)		Liver sample from each day after BDL (days)				Kidney sample from each day after UUO (days)			
Accession #	Name	0	3	7	14	0	3	7	14
Q6ZWV3	60S ribosomal protein L10		+		+			+	
Q99LB2	Dehydrogenase/reductase SDR family member 4		+	+	+		+		
P26040	Ezrin		+				+	+	
Q8R1M2	Histone H2A.J		+						+
Q9D2U9	Histone H2B type 3-A		+	+	+				+
Q921I1	Serotransferrin			+	+				+
P99024	Tubulin beta-5 chain			+	+			+	+

Table 4. The list of overlapped proteins between liver and kidney fibrosis in BPA-incorporated substrates.

TGase (BPA)		Liver sample from each day after BDL (days)				Kidney sample from each day after UUO (days)			
Accession #	Name	0	3	7	14	0	3	7	14
P97351	40S ribosomal protein S3a		+					+	
Q61147	Ceruloplasmin			+					+
P01029	Complement C4-B		+				+	+	+
P11276	Fibronectin		+	+				+	+
Q8BG05	Heterogeneous nuclear ribonucleoprotein A3		+					+	
Q9Z2X1	Heterogeneous nuclear ribonucleoprotein F		+				+	+	+
Q6GSS7	Histone H2A type 2-A				+				+
Q8CGP1	Histone H2B type 1-K				+		+	+	+
P84244	Histone H3.3		+	+			+	+	
P08071	Lactotransferrin		+				+	+	+
P26041	Moesin				+			+	+
P11247	Myeloperoxidase			+					+
Q8VDD5	Myosin-9			+			+	+	+
P62960	Nuclease-sensitive element-binding protein 1			+			+	+	
Q62446	Peptidyl-prolyl cis-trans isomerase FKBP3		+				+	+	
P35700	Peroxiredoxin-1			+			+		
Q61656	Probable ATP-dependent RNA helicase DDX5		+				+	+	+
P31725	Protein S100-A9			+				+	+
Q921I1	Serotransferrin			+			+	+	+
P99024	Tubulin beta-5 chain		+				+	+	+

Figure 1.

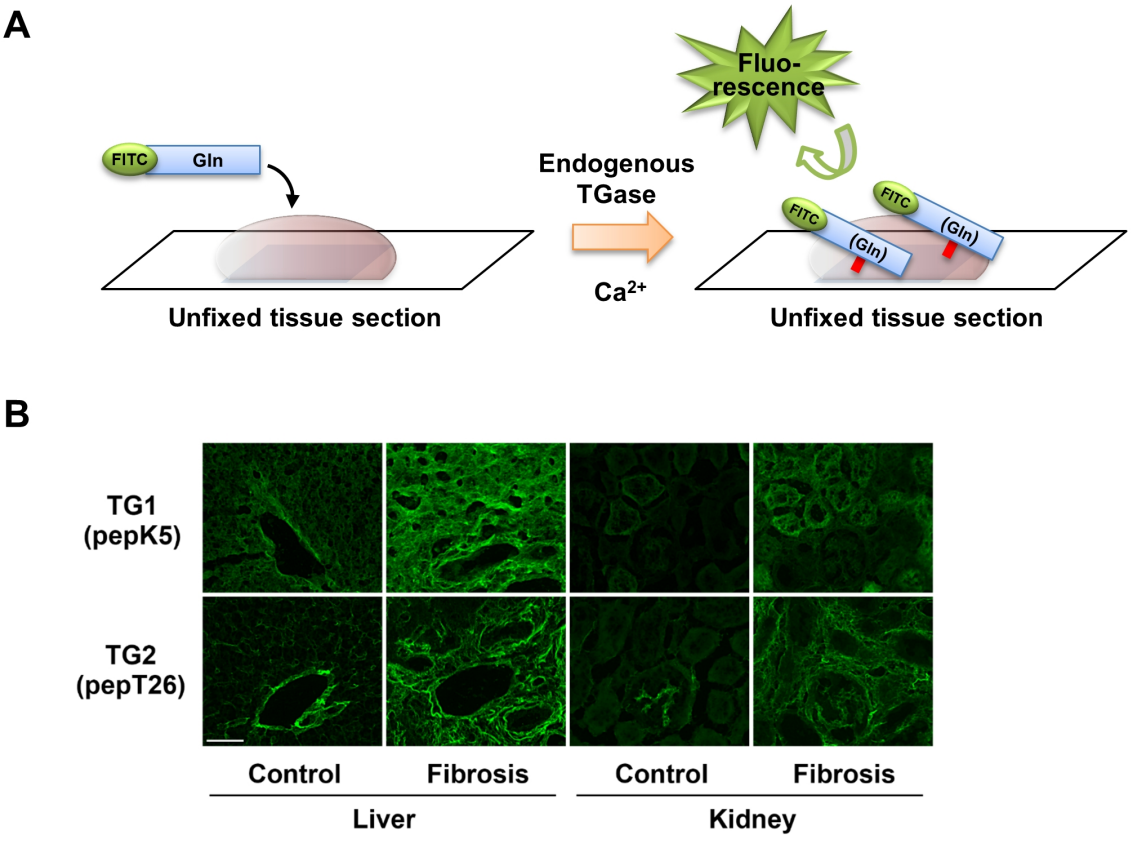
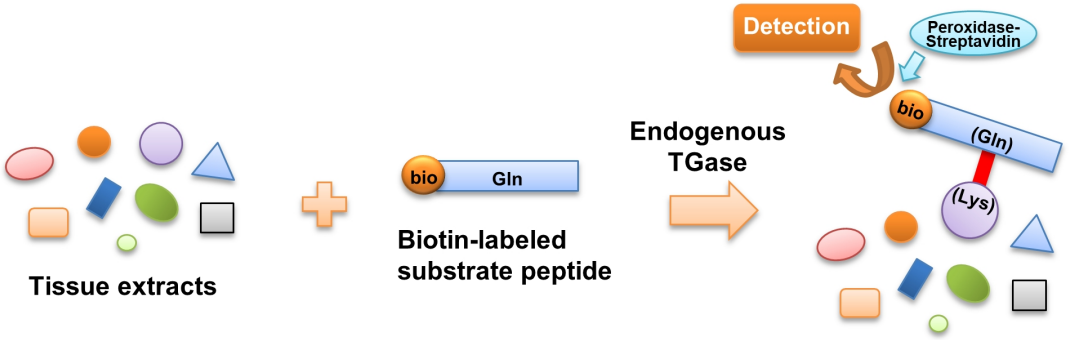

A
Fluo-
rescence
FITC
Gln
Endogenous
TGase
Ca2+
FITC
(Gln)
FITC
(Gln)
Unfixed tissue section
Unfixed tissue section
B
TG1
(pepK5)
TG2
(pepT26)
Control
Fibrosis
Control
Fibrosis
Liver
Kidney

Figure 2.

A


Tissue extracts
bio
Gln
Biotin-labeled
substrate peptide
Endogenous
TGase
Detection
Peroxidase-
Streptavidin
bio
(Gln)
(Lys)

B

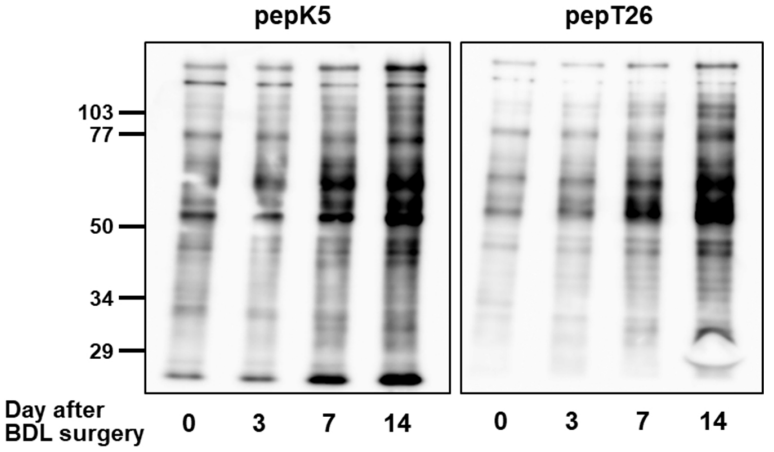

pepK5
pepT26
103
77
50
34
29
Day after
BDL surgery
0
3
7
14
0
3
7
14

Figure 3.

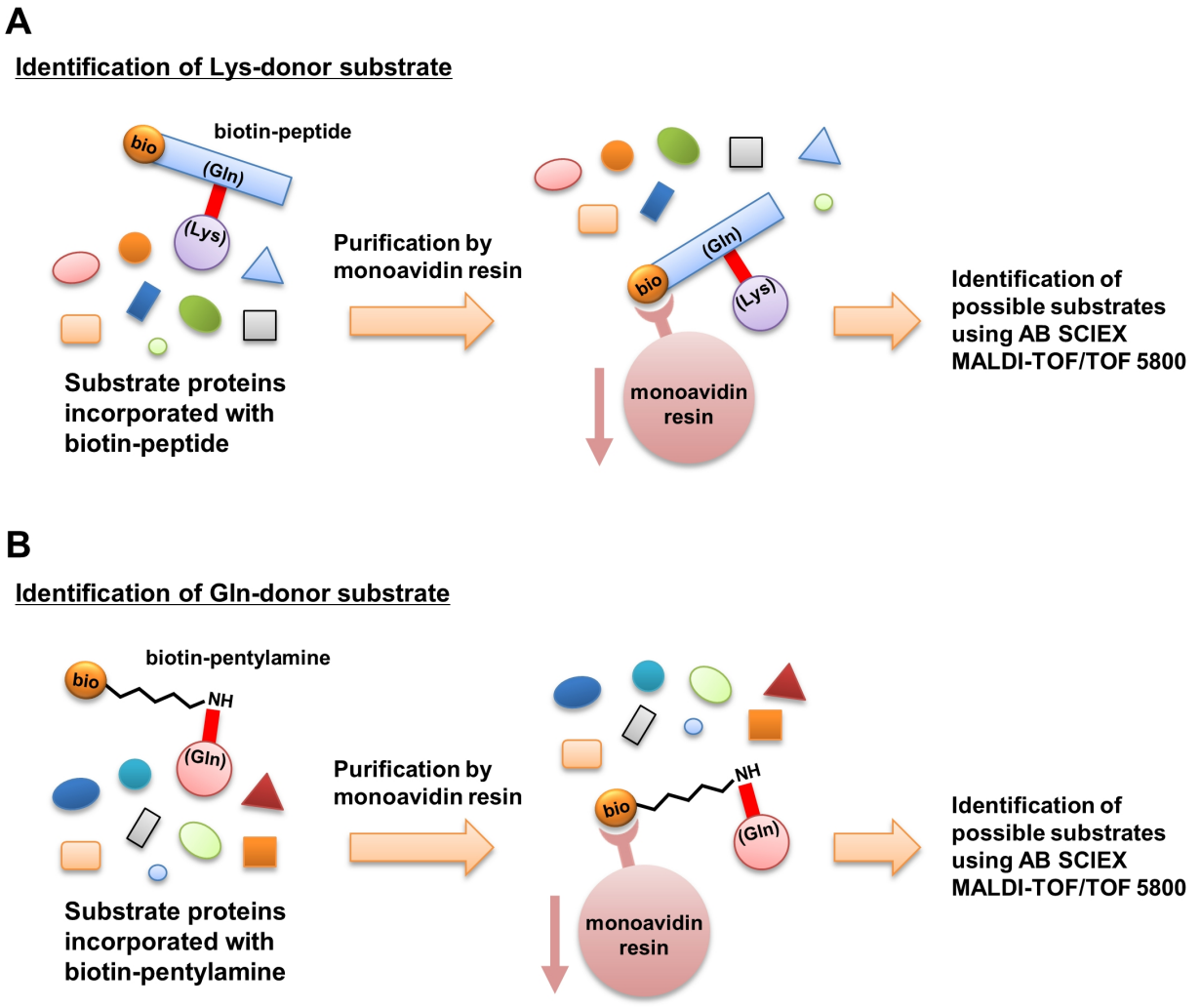
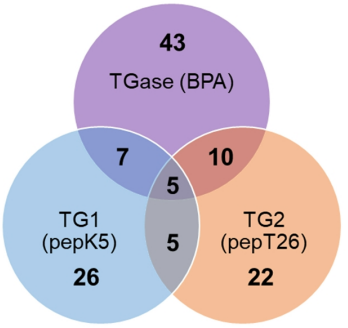

A
Identification of Lys-donor substrate
biotin-peptide
bio
(Gln)
(Lys)
Substrate proteins incorporated with biotin-peptide
Purification by monoavidin resin
(Gln)
bio
(Lys)
monoavidin resin
Identification of possible substrates using AB SCIEX MALDI-TOF/TOF 5800
B
Identification of Gln-donor substrate
biotin-pentylamine
bio
NH
(Gln)
Substrate proteins incorporated with biotin-pentylamine
Purification by monoavidin resin
NH
bio
(Gln)
monoavidin resin
Identification of possible substrates using AB SCIEX MALDI-TOF/TOF 5800

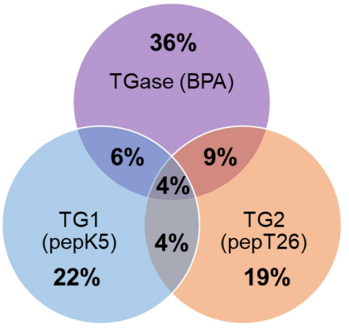
Figure 4.

A Liver fibrosis (BDL)

Identified substrates (#)

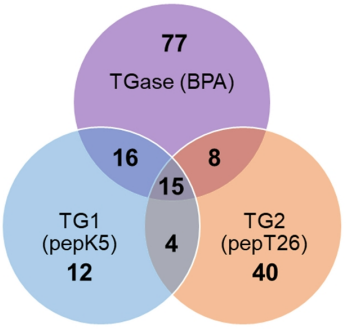


Identified substrates (%)

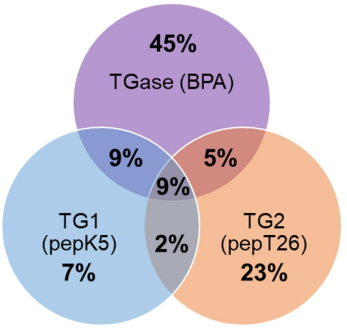


B Kidney fibrosis (UUO)

Identified substrates (#)

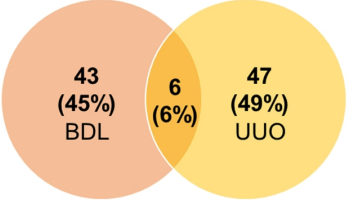


Identified substrates (%)

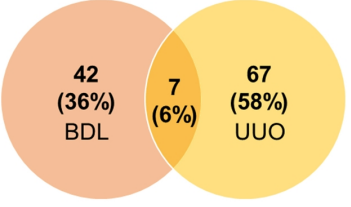


C Identified substrates (# and %)

pepK5



pepT26



BPA