Biosci. Biotechnol. Biochem., **77** (10), 2061–2064, 2013

Immobilization of an Esterase Inhibitor on a Porous Hollow-Fiber Membrane by Radiation-Induced Graft Polymerization for Developing a Diagnostic Tool for Feline Kidney Diseases

Shinya MATSUNO,[1] Daisuke UMENO,[1] Masao MIYAZAKI,[2] Yasuyuki SUZUTA,[3] Kyoichi SAITO,[1] and Tetsuro YAMASHITA[2,†]

[1]*Department of Applied Chemistry and Biotechnology, Chiba University, 1-33 Yayoi, Inage, Chiba 263-8522, Japan*
[2]*Faculty of Agriculture, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan*
[3]*Nippon Zenyaku Kogyo Co., Ltd., 1-1 Tairanoue, Sasagawa, Asaka-machi, Koriyama, Fukushima 963-0196, Japan*

Received May 27, 2013; Accepted July 18, 2013; Online Publication, October 7, 2013
[doi:10.1271/bbb.130428]

Removal of the major urinary protein, cauxin, a carboxylesterase, from cat urine is essential for distinguishing between physiological and abnormal proteinuria by a urine dipstick. We have previously developed a material for removing cauxin by using *lens culinaris agglutinin* (LCA) lectin which targets the *N*-linked oligosaccharides present in cauxin. To improve the affinity and specificity toward cauxin, we immobilized 1,1,1-trifluoro-3-(2-sulfanylethylsulfanyl) propane-2-one, an inhibitor of esterases, to a polymer chain grafted on to a porous hollow-fiber membrane by applying radiation-induced graft polymerization. Normal male urine was forced to permeate through the pores rimmed by the ligand-immobilized polymer chain. Cauxin could not be detected in the effluent from the membrane. The residence time of the urine across a membrane thickness of 1 mm was set at 7 s. The respective dynamic and equilibrium binding capacities of the membrane for cauxin were 2 and 3 mg/g. The developed cauxin-affinity membrane material was more effective for diagnosing cat kidney diseases than the LCA lectin tip.

Key words: radiation-induced graft polymerization; carboxylesterase; cauxin; esterase inhibitor; kidney disease

Kidney diseases are the leading cause of illness and death in aging cats.[1,2] Proteinuria is generally one of the best indicators for the early diagnosis of kidney diseases in medicine.[3] A large excretion of high- and low-molecular-weight (LMW, <60 kDa) proteins respectively indicates glomerular and renal tubular injuries. Detecting proteinuria by using a urine dipstick is used as a convenient and rapid screening method for kidney diseases. However, a urine dipstick is not useful for screening abnormal proteinuria in cats. Cats exhibit proteinuria under both pathological and normal physiological conditions. The urine of normal cats contains cauxin, a 70-kDa carboxylesterase, as the major urinary protein in a species-specific manner.[4] Cauxin is secreted from proximal straight tubular cells into the urine for the production of 2-amino-7-hydroxy-5,5 dimethyl-4-thiaheptanoic acid (felinine), a precursor of species-specific odorants.[5,6] We have previously shown that the excretion level of cauxin decreased in cats with renal diseases due to a decrease in the level of intact renal tubular cells, whereas the excretion levels of albumin and LMW proteins increased.[7] Since tetrabromophenol blue, an active ingredient, coated on a urine dipstick reacts with both urinary albumin and cauxin, the dipstick approach cannot distinguish between physiological and pathological proteinuria in cats.

We have developed a novel method for screening only abnormal proteinuria in cats to overcome this problem.[8] When a urine dipstick exhibits a protein-positive score for urine samples, we recommend using the *lens culinaris agglutinin* (LCA) lectin tip to prepare cauxin-free urine. Since cauxin is a glycoprotein with fucoses that interact with LCA lectin,[9] cauxin is trapped in the tip and removed from the urine. If cauxin-free urine exhibits a protein-positive score, the result indicates that the original urine contained albumin and/or LMW proteins that arise from pathological proteinuria. In contrast, if the cauxin-free urine exhibits a protein-negative score, it proves that the original urine sample contained a large amount of cauxin which indicates physiological proteinuria. We have demonstrated in several clinical samples that the LCA lectin tip was useful for screening abnormal proteinuria in cats. Nonetheless, we also recognized the importance of improving the affinity and specificity of the removal material toward cauxin, because the current LCA lectin tip cannot completely remove cauxin from cat urine. The lectin tip also removes small amounts of serum proteins, immunoglobulin G and unidentified LMW proteins.

We aimed in this study to develop a novel material for removing cauxin from cat urine. We focused on using trifluoroketone (TFK) as a ligand of cauxin. TFK is an inhibitor of carboxylesterases by binding to the substrate pocket.[10] Since TFK exhibits high affinity towards cauxin, it was hypothesized that a material with TFK could specifically capture cauxin in cat urine without binding with other proteins. We have previously immobilized TFK derivatives on the surface of polymer

† To whom all correspondence should be addressed. Tel: +81-19-621-6157; Fax: +81-19-621-6154; E-mail: yamashit@iwate-u.ac.jp
Abbreviations: LCA, *lens culinaris agglutinin*; TFK, trifluoroketone; GMA, glycidyl methacrylate; DEV, dimensionless effluent volume

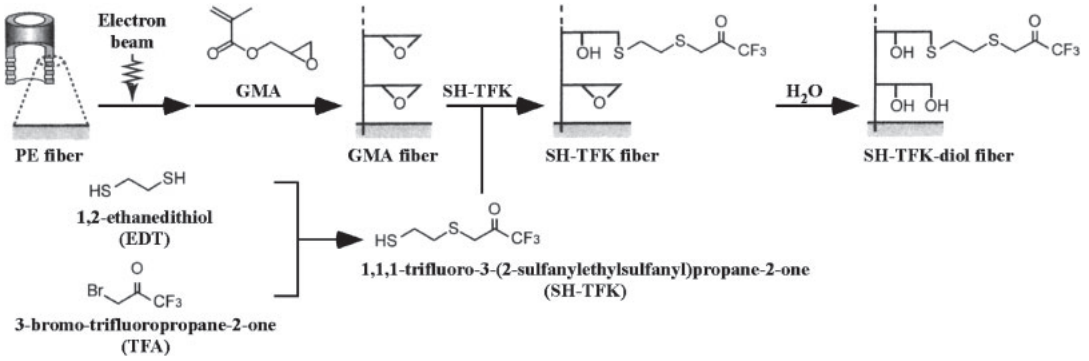

Fig. 1. Preparation Scheme for the TFK Immobilized Hollow-Fiber Membrane by Radiation-Induced Graft Polymerization and Subsequent Chemical Modification.

substrates by using radiation-induced graft polymerization.[11] The novel materials resulting from this work were then tested to determine whether they could completely remove cauxin from cat urine.

Materials and Methods

Materials. A porous hollow-fiber membrane made of high-density polyethylene (HDPE) was supplied by Asahi Kasei Chemicals Co. and used as a trunk polymer for grafting. The hollow fiber had an internal diameter of 2 mm with a thickness of 0.5 mm. The respective porosity and average pore diameter of this hollow fiber were 70% and 0.4 μm. The epoxy-group-containing vinyl monomer, glycidyl methacrylate (GMA, $CH_2{=}CCH_3COOCH_2CHOCH_2$), was purchased from Nakalai Tesque Co. for use as a reactive monomer. 1,2-Ethanedithiol (C2DT) and 3-bromo-trifluoropropane-2-one (TFA) were respectively purchased from Tokyo Kasei Industry and Sigma-Aldrich. All other chemicals were of analytical grade or higher. A urine model for cats with renal diseases, in which albumin and cauxin were detected as the major proteins, was prepared. Urine samples were first collected from three experimental male cats with clinically normal history at Nippon Zenyaku Kogyo Co. Equal volumes of each urine sample were mixed, and then bovine serum albumin (BSA) was spiked into the mixture at a final concentration of 0.2 mg/mL.

Preparation of affinity porous membranes in a hollow-fiber form. The preparation scheme for the affinity porous hollow-fiber membrane followed the four steps shown in Fig. 1: (i) irradiation by an electron beam of the porous hollow-fiber membrane, (ii) grafting of GMA on to the porous hollow-fiber membrane, (iii) addition of 1,1,1-trifluoro-3-(2-sulfanylethylsulfnanyl)propane-2-one (SH-TFK) to some of the epoxy groups, and (iv) addition of water to the remaining epoxy groups. Briefly, the porous hollow-fiber membrane was irradiated with a 200 kGy electron beam. GMA was then graft-polymerized on to the porous hollow-fiber membrane, the resulting porous hollow-fiber membrane being referred to as a GMA fiber. The degree of grafting (dg) is defined as follows:

$$\text{dg}\ (\%) = 100(W_1 - W_0)/W_0$$

where W_0 and W_1 are the respective masses of the trunk and GMA fibers.

SH-TFK was synthesized by reacting 1,2-ethanedithiol with 3-bromo-trifluoropropane-2-one for 48 h at room temperature. A fraction of the produced epoxy groups of the grafted polymer chains on dg 130% of the GMA fibers were converted into affinity ligands by the reaction with SH-TFK. The molar conversion of the epoxy groups into specific affinity ligands for cauxin was evaluated from the mass gain of the membrane by the following equation:

$$\text{molar conversion}\ (\%) = 100[(W_2 - W_1)/204]/[(W_1 - W_0)/142]$$

where W_2 is the mass of the SH-TFK fiber, and 204 and 142 are the respective molecular masses of SH-TFK and GMA. The remaining epoxy groups were finally converted into diol groups by the reaction with water in 0.5 M H_2SO_4.

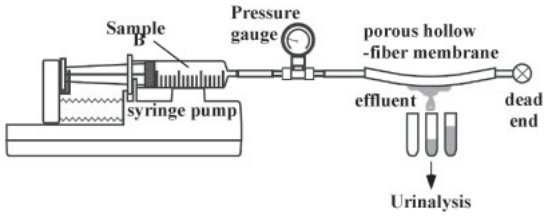

Fig. 2. Experimental Apparatus for Permeation of the Feed Solution Thorough Pores of the TFK Immobilized Hollow-Fiber Membrane.

Removal of cauxin by using the affinity porous membrane in the permeation mode. The experimental apparatus for removing cauxin from male cat urine is illustrated in Fig. 2. The 2.5-cm-long SH-TFK fiber was arranged in an I-shaped configuration. One end of the SH-TFK fiber was connected to a syringe, and the other end of the fiber was sealed. Clinically normal male cat urine or model urine representing a renal disease was fed to the inside surface of the fiber and permeated through the pores by a syringe pump. The permeation rate ranged from 2 to 30 mL/h. The effluent penetrating the outside surface of the fiber was continuously sampled with fraction vials. The protein concentration was determined by the Bradford method.

The components of the proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) under non-reduced conditions. Proteins were detected by Coomassie brilliant blue R-250 (CBB).

Results and Discussion

Affinity ligand density

We used in this study the radiation-induced graft polymerization technique to give better cauxin removal than the LCA lectin tip by increasing the affinity and specificity toward cauxin. Radiation-induced graft polymerization has previously been used to prepare porous adsorbents for recovering or purifying proteins based on various ion-exchange, hydrophobic and affinity interactions.[11] For example, a porous hollow-fiber membrane that is industrially used for microfiltration has been modified into an anion-exchange hollow-fiber membrane.[12,13] A protein solution was induced to permeate through the pores of the membrane to minimize the diffusional mass-transfer resistance of the protein to an ion-exchange group. A ligand of cauxin was therefore immobilized to the polymer chain grafted on to a porous hollow fiber membrane, and the removal rate of cauxin from cat urine during permeation of the solution through the pores was examined.

Figure 3 shows the time-course characteristics for molar conversion of the epoxy group into the SH-TFK

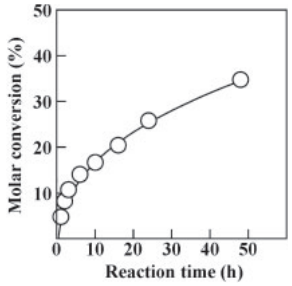

Fig. 3. Time-Course Characteristics for Molar Conversion of the Epoxy Group into the SH-TFK Ligand.

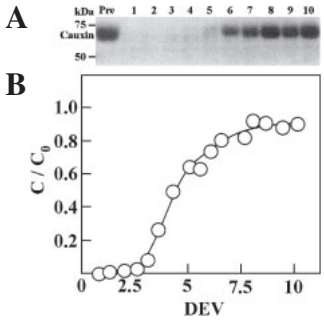

Fig. 4. Analysis of Protein Components in the Effluent of an SH-TFK Fiber in Normal Urine of the Male Cat.

A, SDS–PAGE with CBB staining. Lanes correspond to a series of effluent fractions from the fiber. B, Breakthrough curves of the fiber for cauxin. C_0 and C are the respective protein concentrations of the feed and effluent.

ligand. The molar conversion increased as the reaction time increased, the molar conversion reaching 70% after a reaction time of 5 h, this being equivalent to 2 mmol/g. This ligand density was comparable to that of a commercially available affinity adsorbent. We suggest that a trifluoroketone moiety would be easily immobilized to supporting materials with sufficient density because of its low molecular mass.

Removal of cauxin from normal cat urine by the affinity membrane

The breakthrough curve of the SH-TFK fiber for cat urine is shown in Fig. 4B. Breakthrough curves, *i.e.*, the changes in total concentration of the protein as a function of the cumulative effluent volume, were determined together with a protein analysis by SDS–PAGE (Fig. 4A). The ordinate in Fig. 4A is the relative protein concentration of the effluent to that of the feed and the abscissa is the dimensionless effluent volume (DEV) defined by the following equation:

$$\text{DEV [x]} = (\text{effluent volume}) / (\text{membrane volume including the lumen part})$$

Irrespective of the value for x, the breakthrough curves overlapped, making identical equilibrium binding capacity apparent.

Greater than 90% of cauxin was removed from the urine when a residence time of 7 s was used across a membrane thickness of 1 mm. In previous studies, a smear band of cauxin was detected in the effluent of urine passed through the LCA tip. In contrast, no cauxin

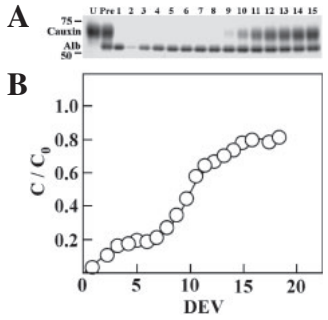

Fig. 5. Analysis of Protein Components in the Effluent of an SH-TFK Fiber for the Urine Model of Cats with Renal Diseases Containing 0.2 mg/mL of BSA.

A, SDS–PAGE with CBB staining. Lanes correspond to a series of effluent fractions from the fiber. B, Breakthrough curves of the fiber for cauxin. C_0 and C are the respective protein concentrations of the feed and effluent.

band could be detected in the effluent from the affinity membrane in this study.

The results of this study demonstrate that the density was sufficient to remove cauxin from cat urine. It was also expected that the material could be preserved at room temperature, because the TFK ligand is stable, in contrast to LCA lectin that must be kept at 4 °C. These results indicate that the novel material for removing cauxin from cat urine was better than the LCA lectin tip in terms of its affinity and storage temperature.

Removal of cauxin from a renal disease urine model by the affinity membrane

The breakthrough curve of the SH-TFK fibers for a urine model representing renal disease are shown in Fig. 5B, together with the SDS–PAGE analysis in Fig. 5A. The fraction numbers in these figures are identical to those indicated in the breakthrough curve. SDS–PAGE for the urine model detected only two major bands, cauxin and spiked albumin (lane 2). In contrast, after treating the urine model with the SH-TFK fiber, no cauxin could be detected in the effluent until DEV of 3, although albumin remained constant throughout the adsorption (lanes 2–8).

The breakthrough curves for the SH-TFK fiber shown in Fig. 6 are at various flow rates for the kidney disease urine model. In the range of 2–30 mL/h, the breakthrough curves overlap irrespective of the flow rate or the residence time of the liquid through the pores. This indicates that the diffusional mass-transfer resistance of cauxin from the pore interior to the graft chain was negligible and that the affinity interaction between cauxin and the SH-TFK ligand was instantaneous. These adsorption characteristics are favorable for rapidly removing cauxin from the urine.

Although our previous study has demonstrated that an LCA lectin tip was useful for screening abnormal proteinuria in cats with renal diseases, we also found that LCA lectin captured not only cauxin, but also immunoglobulin G and a few unidentified LMW proteins which had fucose on an *N*-linked oligosaccharide.[8] This present study suggests that TFK was a better ligand for cauxin than LCA lectin in terms of the specific removal of cauxin from cat urine. Based on our previous finding that no esterase other than cauxin was

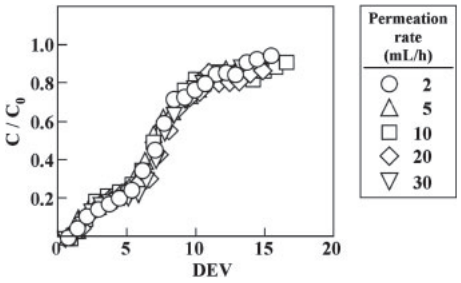

Fig. 6. Breakthrough Curves of an SH-TFK Fiber for Protein in a Renal Disease Urine Model Containing 0.2 mg/mL of BSA.

C_0 and C are the respective protein concentrations of the feed and effluent.

excreted into cat urine,[4] TFK should remove only cauxin from cat urine. We will go to next step in which clinical urine samples collected from cats with renal diseases will test whether the SH-TFK fiber is a useful material for screening abnormal proteinuria with higher accuracy than the LCA lectin tip.

In conclusion, the technique for radiation-induced graft polymerization was applied to develop a diagnostic tool for kidney diseases in the domestic cat. A novel porous adsorbent capable of specifically capturing cauxin was prepared based on affinity interaction, *i.e.*, the interaction between an enzyme and inhibitor. 1,1-Trifluoro-3-(2-sulfanylethylsulfanyl) propane-2-one as an inhibitor of cauxin was immobilized to a polymer chain grafted on to the pore surface of a porous hollow-fiber membrane. Over 90% of cauxin was removed during the permeation of normal male urine through the affinity porous hollow-fiber membrane, and a urine dipstick distinguished between normal and abnormal proteinuria. A kit designed for removing cauxin is inexpensive to produce and easy to use for both veterinarians and the owners of cats, and we believe that this would be a useful screening method for abnormal proteinuria in cats.

Acknowledgments

The authors thank Dr. Noboru Kubota of Asahi Kasei Chemicals Co. for providing the starting porous hollow-fiber membrane. This study was supported by the A-step feasibility study program (02-038) of Japan Science and Technology Agency (JST).

References

1) DiBartola SP, Rutgers HC, Zack PM, and Tarr MJ, *J. Am. Vet. Med. Assoc.*, **190**, 1196–1202 (1987).
2) Elliott J and Barber PJ, *J. Small Anim. Pract.*, **39**, 78–85 (1998).
3) D'Amico G and Bazzi C, *Kidney Int.*, **63**, 809–825 (2003).
4) Miyazaki M, Kamiie K, Soeta S, Taira H, and Yamashita T, *Biochem. J.*, **370**, 101–110 (2003).
5) Miyazaki M, Yamashita T, Hosokawa M, Taira H, and Suzuki A, *Comp. Biochem. Physiol. B Biochem. Mol. Biol.*, **145**, 270–277 (2006).
6) Miyazaki M, Yamashita T, Suzuki Y, Saito Y, Soeta S, Taira H, and Suzuki A, *Chem. Biol.*, **13**, 1071–1079 (2006).
7) Miyazaki M, Soeta S, Yamagishi N, Taira H, Suzuki A, and Yamashita T, *Res. Vet. Sci.*, **82**, 76–79 (2007).
8) Miyazaki M, Fujiwara K, Suzuta Y, Wakamatsu N, Taira H, Suzuki A, and Yamashita T, *Vet. J.*, **189**, 312–317 (2011).
9) Suzuki Y, Miyazaki M, Ito E, Suzuki M, Yamashita T, Taira H, and Suzuki A, *Biosci. Biotechnol. Biochem.*, **71**, 811–816 (2007).
10) Ashour MB and Hammock BD, *Biochem. Pharmacol.*, **36**, 1869–1879 (1987).
11) Nasefa MM and Hegazyb E-SA, *Prog. Polym. Sci.*, **29**, 499–561 (2004).
12) Hagiwara K, Yonedu S, Saito K, Shiraishi T, Sugo T, Tojyo T, and Katayama E, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **821**, 153–158 (2005).
13) Abrol K, Qazi GN, and Ghosh AK, *J. Biotechnol.*, **128**, 838–848 (2007).