Immobilization of *Thermoanaerobacterium xylanolyticum* *Tx*GH116 and E441G nuclophile mutant

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Abstract

Thermoanaerobacterium xylanolyticum *Tx*GH116 β-glucosidase belongs to glycoside hydrolase (GH) family 116 and hydrolyzes β-1,3- and β-1,4- linked oligosaccharides. *Tx*GH116 E441G nucleophile mutant catalyzed transfer of glucose from α-glucosyl fluoride (α-GlcF) to cellobiose acceptor without hydrolysis of the products and showed broad specificity for α-glycosyl fluoride donors and *p*-nitrophenyl glycoside acceptors. Moreover, the *Tx*GH116 E441G catalyzed synthesis of α-D-glucosyl azide from sodium azide and *p*NP-β-D-glucoside (*p*NPGlc) or cellobiose for production of α-glucosyltriazoles. Enzyme immobilization promotes high catalytic activity and stability, and convenient handling of reusable enzymes. In order to economize on the enzymes, *Tx*GH116 and E441G were immobilized on Sepharose 4B activated by cyanogen bromide. The optimum pH of *Tx*GH116 was slightly reduced from 5.5 to 5.0 upon immobilization and had lower relative activity than free enzyme from pH 5.5 to 8.0, while the temperature optimum and thermal stability of immobilized *Tx*GH116 was similar to free enzyme. The immobilized *Tx*GH116 remained 94% of its initial activity after 20 cycles. The optimum pH of immobilized E441G was pH 4.5, similar to free enzyme, while immobilized E441G had lower thermal stability than free enzyme. The activity of immobilized E441G remained only 53% after 10 cycles. Small scale immobilization of E441G on immobilized metal affinity chromatography (IMAC) resin gave higher stability than on cyanogen bromide-activated Sepharose 4B, with 100% activity remaining after 10 cycles, but it was unstable in larger scale reactions. Therefore, the immobilization process could not enhance the pH and temperature stability of *Tx*GH116 and E441G, but the immobilized enzymes were reusable.

Introduction

Immobilization of enzymes on solid supports is a widespread technology to achieve more stable, active and reusable enzymes and used in numerous applications, including biosensors, food packaging materials, and biofuel production.[1,2] The thermophilic bacteria β-glucosidase from *Thermoanaerobacterium xylanolyticum* (*Tx*GH116) has broad substrate specificity.[3,4] Several structures of *Tx*GH116 alone and in complex with its inhibitors have been determined.[4-7] The *Tx*GH116 E441G nucleophile mutant exhibited highest glycosynthase activity to transfer glucose from α-fluoroglucoside (α-GlcF) to cellobiose acceptor and catalyzed synthesis of α-D-glucosyl azide from sodium azide and *p*NP-β-D-glucoside (*p*NPGlc) and cellobiose in chemical rescue experiment (**Fig. 1**) for production of α-glucosyltriazoles.[8,9] Combining structures of *Tx*GH116 E441A with α-GlcF donor and E441A or E441G with cellobiose acceptor provides a plausible structure of the catalytic ternary complex.[8] Here, we report the immobilization of *Tx*GH116 and E441G on CNBr-activated Sepharose 6B (covalent binding) and immobilized metal affinity chromatography (IMAC, non-covalent binding) to pH and temperature stabilities and catalytic activity. It was shown that immobilization process could not enhance the pH and temperature stability of *Tx*GH116 and E441G, but the immobilized enzymes were reusable.

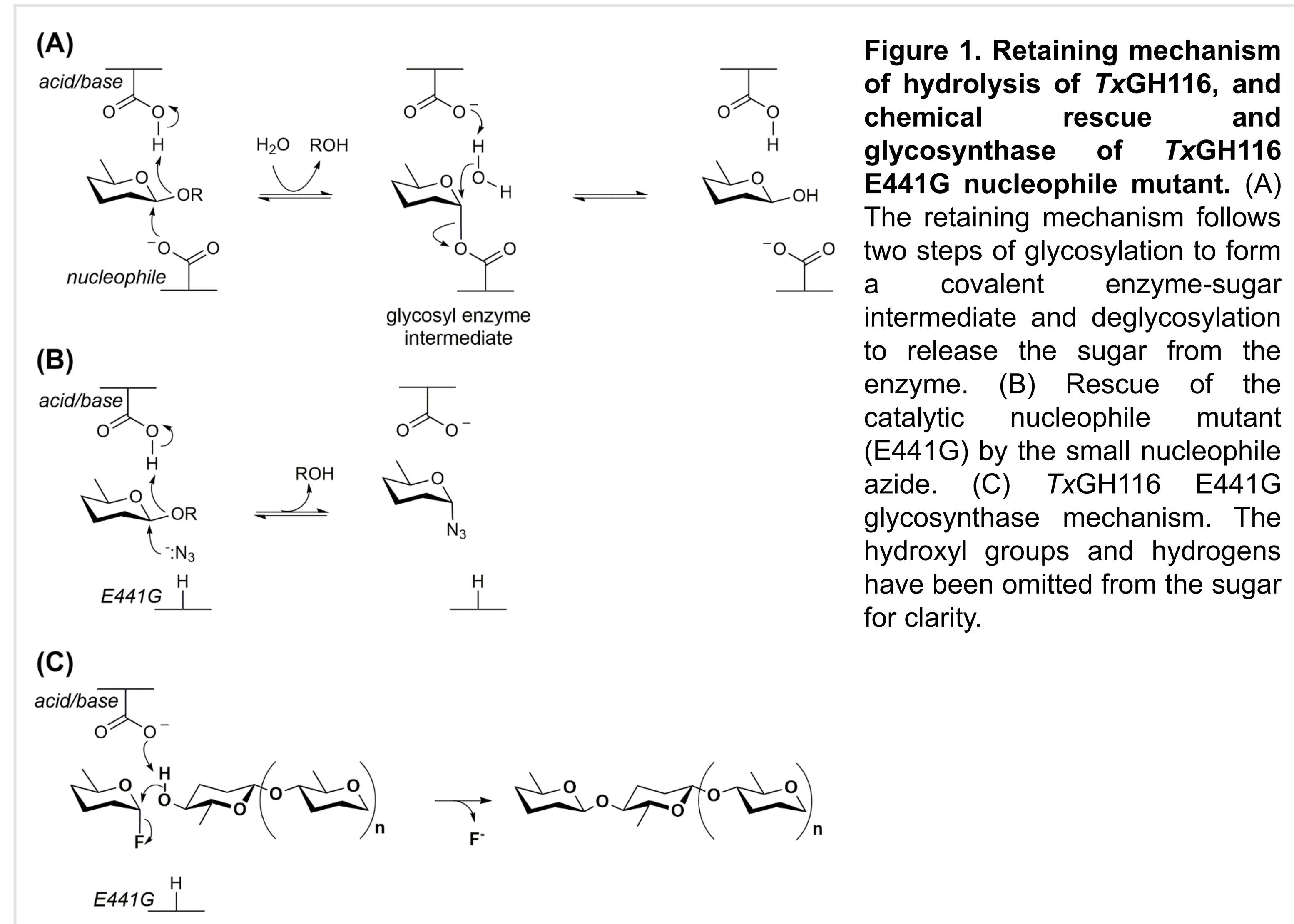

Figure 1. Retaining mechanism of hydrolysis of *Tx*GH116, and chemical rescue and glycosynthase of *Tx*GH116 E441G nucleophile mutant. (A) The retaining mechanism follows two steps of glycosylation to form a covalent enzyme-sugar intermediate and deglycosylation to release the sugar from the enzyme. (B) Rescue of the catalytic nucleophile mutant (E441G) by the small nucleophile azide. (C) *Tx*GH116 E441G glycosynthase mechanism. The hydroxyl groups and hydrogens have been omitted from the sugar for clarity.

Results

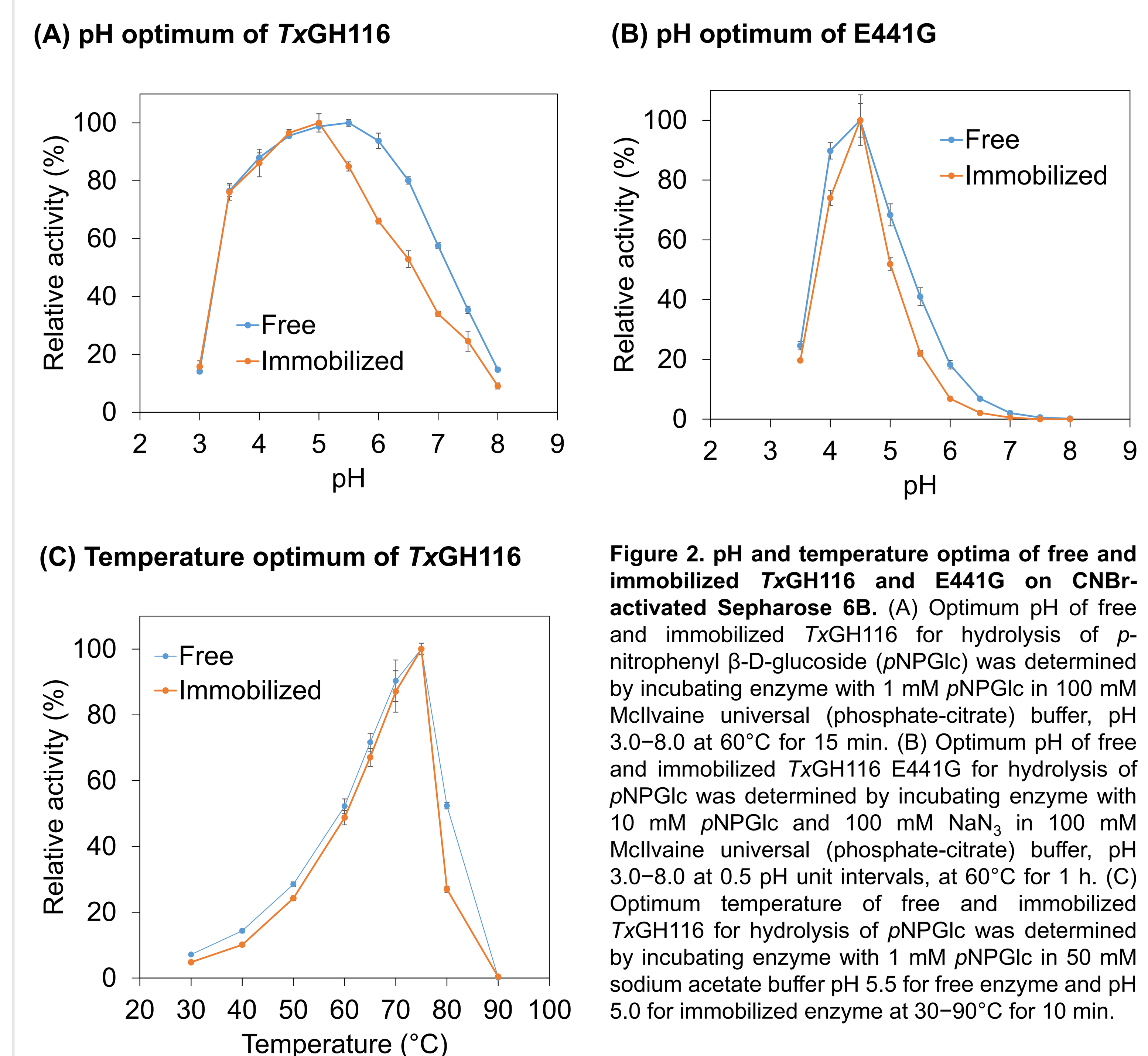

Figure 2. pH and temperature optima of free and immobilized *Tx*GH116 and E441G on CNBr-activated Sepharose 6B. (A) Optimum pH of free and immobilized *Tx*GH116 for hydrolysis of *p*-nitrophenyl β-D-glucoside (*p*NPGlc) was determined by incubating enzyme with 1 mM *p*NPGlc in 100 mM McIlvaine universal (phosphate-citrate) buffer, pH 3.0−8.0 at 60°C for 15 min. (B) Optimum pH of free and immobilized *Tx*GH116 E441G for hydrolysis of *p*NPGlc was determined by incubating enzyme with 10 mM *p*NPGlc and 100 mM NaN_3 in 100 mM McIlvaine universal (phosphate-citrate) buffer, pH 3.0−8.0 at 0.5 pH unit intervals, at 60°C for 1 h. (C) Optimum temperature of free and immobilized *Tx*GH116 for hydrolysis of *p*NPGlc was determined by incubating enzyme with 1 mM *p*NPGlc in 50 mM sodium acetate buffer pH 5.5 for free enzyme and pH 5.0 for immobilized enzyme at 30−90°C for 10 min.

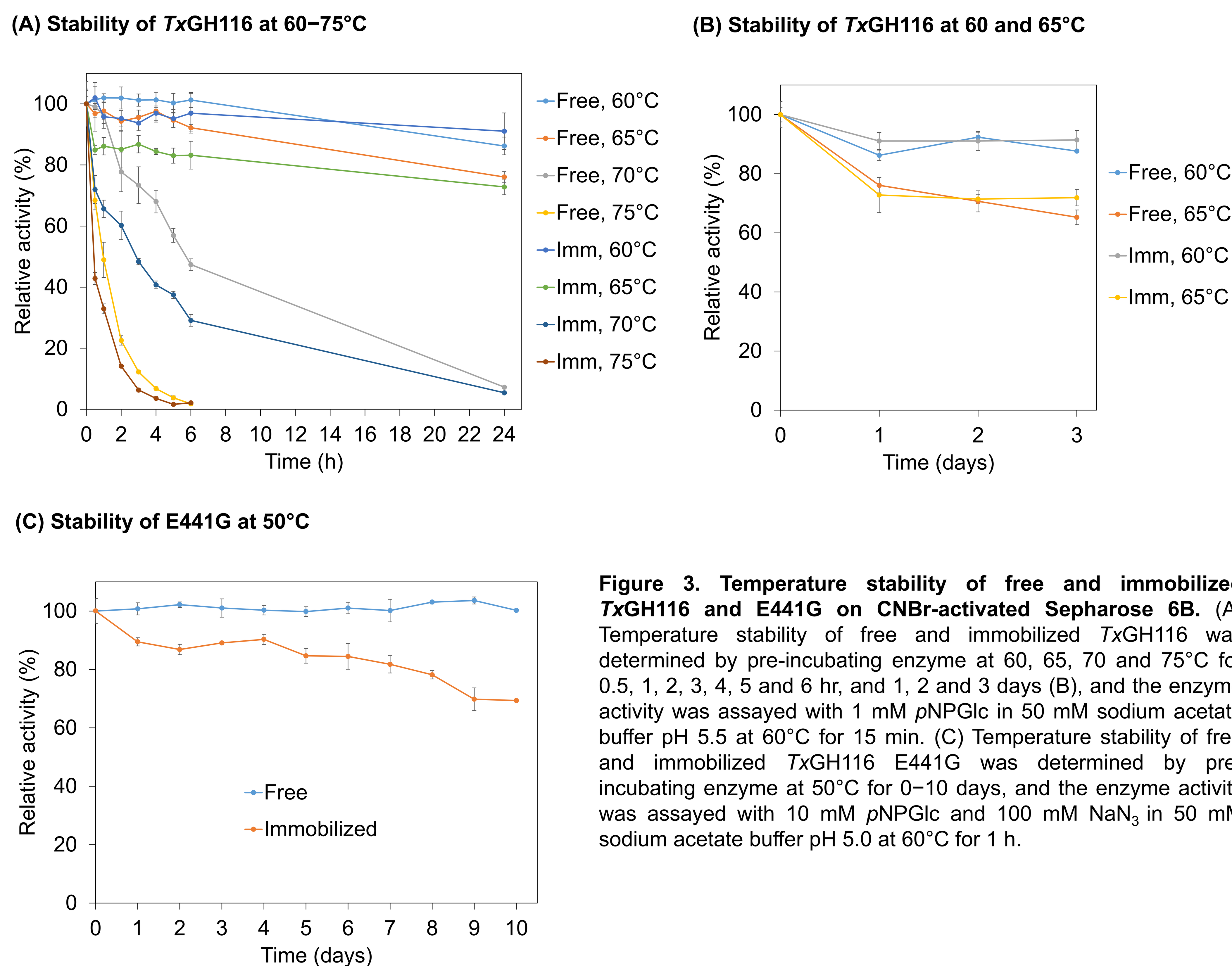

Figure 3. Temperature stability of free and immobilized *Tx*GH116 and E441G on CNBr-activated Sepharose 6B. (A) Temperature stability of free and immobilized *Tx*GH116 was determined by pre-incubating enzyme at 60, 65, 70 and 75°C for 0.5, 1, 2, 3, 4, 5 and 6 hr, and 1, 2 and 3 days (B), and the enzyme activity was assayed with 1 mM *p*NPGlc in 50 mM sodium acetate buffer pH 5.5 at 60°C for 15 min. (C) Temperature stability of free and immobilized *Tx*GH116 E441G was determined by pre-incubating enzyme at 50°C for 0−10 days, and the enzyme activity was assayed with 10 mM *p*NPGlc and 100 mM NaN_3 in 50 mM sodium acetate buffer pH 5.0 at 60°C for 1 h.

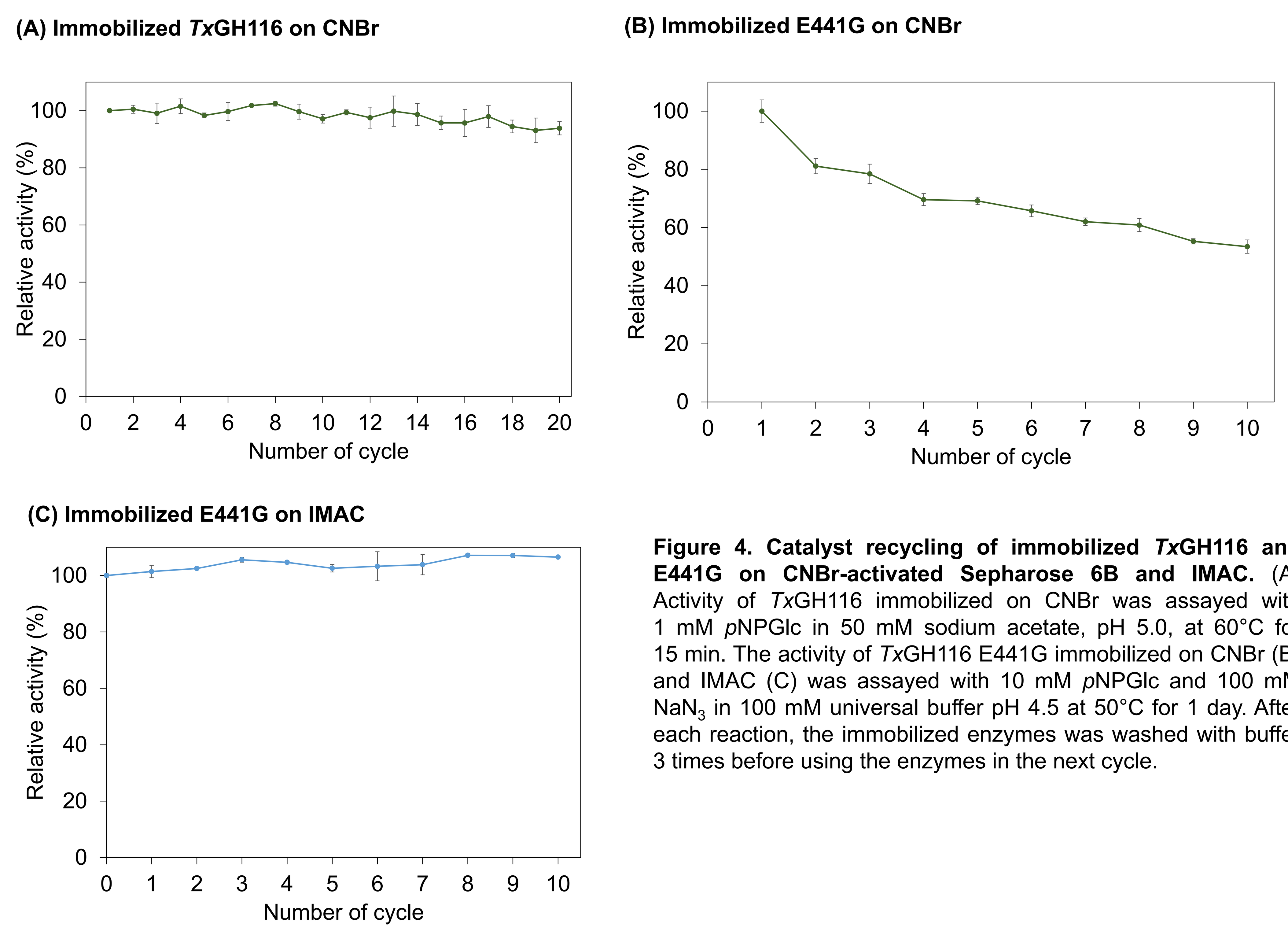

Figure 4. Catalyst recycling of immobilized *Tx*GH116 and E441G on CNBr-activated Sepharose 6B and IMAC. (A) Activity of *Tx*GH116 immobilized on CNBr was assayed with 1 mM *p*NPGlc in 50 mM sodium acetate, pH 5.0, at 60°C for 15 min. The activity of *Tx*GH116 E441G immobilized on CNBr (B) and IMAC (C) was assayed with 10 mM *p*NPGlc and 100 mM NaN_3 in 100 mM universal buffer pH 4.5 at 50°C for 1 day. After each reaction, the immobilized enzymes was washed with buffer 3 times before using the enzymes in the next cycle.

Conclusions

Immobilization of TxGH116 and E441G nucleophile mutant on CNBr-activated Sepharose 6B and IMAC gave activities with similar pH and temperature optima and similar or lower stability compared to free enzymes. The immobilized enzymes were reusable for 10-20 cycles, depending on the variant and immobilization chemistry.

References

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Acknowledgements

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