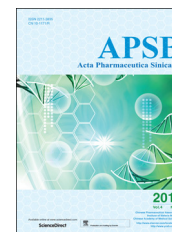




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ORIGINAL ARTICLE

Establishment of a selective evaluation method for DPP4 inhibitors based on recombinant human DPP8 and DPP9 proteins



Jinglong Liu, Yi Huan, Caina Li, Minzhi Liu, Zhufang Shen*

State Key Laboratory of Bioactive Substances and Functions of Natural Medicines, Diabetes Research Center, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China

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Abstract Dipeptidyl peptidase 4 (DPP4) is recognised as an attractive anti-diabetic drug target, and several DPP4 inhibitors are already on the market. As members of the same gene family, dipeptidyl peptidase 8 (DPP8) and dipeptidyl peptidase 9 (DPP9) share high sequence and structural homology as well as functional activity with DPP4. However, the inhibition of their activities was reported to cause severe toxicities. Thus, the development of DPP4 inhibitors that do not have DPP8 and DPP9 inhibitory activity is critical for safe anti-diabetic therapy. To achieve this goal, we established a selective evaluation method for DPP4 inhibitors based on recombinant human DPP8 and DPP9 proteins expressed by Rosetta cells. In this method, we used purified recombinant 120 kDa DPP8 or DPP9 protein from the Rosetta expression system. The optimum concentrations of the recombinant DPP8 and DPP9 proteins were 30 ng/mL and 20 ng/mL, respectively, and the corresponding concentrations of their substrates were both 0.2 mmol/L. This method was highly reproducible and reliable for the evaluation of the DPP8 and DPP9 selectivity for DPP4 inhibitor candidates, which would provide valuable guidance in the development of safe DPP4 inhibitors.

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*Corresponding author. Tel./fax: +86 10 8317 2669.

E-mail address: shenzhf@imm.ac.cn (Zhufang Shen).

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1. Introduction

Dipeptidyl peptidase 8 (DPP8) and dipeptidyl peptidase 9 (DPP9) belong to the S9b gene family (also called the ‘DPP4 gene family’), which share sequence homology, a similar tertiary structure and functional activity with dipeptidyl peptidase 4 (DPP4)¹. Considering the post-proline dipeptidyl aminopeptidase activity of the S9b gene family, various neuropeptides, chemokines, and peptide hormones can be their natural substrates and be involved in their protein degradation². Therefore, many of these enzymes have been found to be effective drug targets. For example, DPP4 is a target of type 2 diabetes. By extending the half-life of endogenous glucagon-like peptide-1 (GLP-1), DPP4 inhibitors can induce insulin secretion, lower blood glucose and HbA_{1c} levels, reduce the apoptosis and improve the proliferation of β cells. Five DPP4 inhibitors, sitagliptin, valdagliptin, saxagliptin, alogliptin and linagliptin, have been on the market³.

The safety or side effects of DPP4 inhibitors are the key points of consideration during drug discovery, mainly due to the similarity of DPP8/9 with DPP4. The amino acid sequences of DPP8 and DPP9 are highly identical to DPP4^{4,5}. All three proteins also share similar structures, consisting of an N-terminal, eight-bladed β -propeller, C-terminal α/β hydrolase domain⁶, a catalytic triad (Ser–Asp–His) and a conserved Glu motif (Glu–Glu)⁷. Currently, little is known about the endogenous substrates and biological functions of DPP8 and DPP9, but some studies have indicated that selective DPP8 and DPP9 inhibitors may cause severe toxicities, such as thrombocytopenia, alopecia, reticulocytopenia, enlarged lymph nodes, splenomegaly, multiorgan histopathological changes and mortality in rats and gastrointestinal toxicity in dogs. Inhibition of DPP8 and DPP9 activities may attenuate T cells activation and proliferation *in vitro*⁸. So without DPP8 and DPP9 inhibitory activity is very important to the development of a DPP4 inhibitor as an anti-diabetic agent.

This study was to establish an evaluation method based on recombinant human DPP8 and DPP9 proteins to detect the selectivity of DPP4 inhibitor candidates, which would provide valuable guidance in the development of safe DPP4 inhibitors.

2. Materials and methods

2.1. Chemicals and reagents

The recognised dipeptidyl peptidase (DPP) substrate Gly-Pro-*p*-nitroanilide and DPP4 enzyme were purchased from Sigma (Missouri, USA). The VigoScript first strand cDNA synthesis kit was a product of Vigorous (Beijing, China). Rosetta competent cells were obtained from TransGen (Beijing, China). Restriction enzymes were purchased from Takara (Dalian, China). Nickel affinity columns, Trizol reagent and DNA polymerase were obtained from Invitrogen (Shanghai, China). The plasmid vector pET32-a(+) was a product of Novagen (Shanghai, China). The anti-His antibody was purchased from Abmart (Shanghai, China). The DPP inhibitor compounds, sitagliptin and UAMC00132 were provided by Prof. Haihong Huang.

2.2. Cloning and construction of recombinant DPP8 and DPP9

The full-length fragments of human DPP8 and DPP9 were obtained *via* reverse-transcription (RT)–PCR technology from HeLa and HepG2 cells, respectively. Primers for human DPP8 (GenBank ID: AF221634;

DPP8-Forward-*Hind* III 5′-ATCAAGCTTGCCACCATGGCAGC-AGCAATG-3′ and DPP8-Reverse-*Sal* I 5′-ATCGTCGACTTATA-TCACTTTTAGAGCAGCAATACG-3′) and DPP9 (GenBank ID: AF374518; DPP9-Forward-*Bam*H I 5′-ATCGGATCCGCCACCAT-GGGGAAGGTAAAG-3′ and DPP9-Reverse-*Xho* I 5′-ATCCTCGA-GTCAGAGGTATTCCTGTAGAAAGTGCAG-3′) were designed with restriction enzyme sites for directional cloning. The PCR was performed as follows: DNA denaturation at 94 °C for 10 min, then 35 cycles (94 °C for 10 s, 65.8 °C for 30 s, 72 °C for 3 min) and a final extension at 72 °C for 10 min. The *Hind* III/*Sal* I fragment of DPP8 was subcloned into the *Hind* III and *Xho* I digested pET32-a(+) expression vector, generating plasmid pET32-a(+)-DPP8. Meanwhile, the *Bam*H I/*Xho* I fragment of DPP9 was subcloned into the *Bam*H I and *Xho* I digested pET32-a(+) expression vector, generating plasmid pET32-a(+)-DPP9. Finally, their sequences were verified. pET32-a(+)-DPP8 and pET32-a(+)-DPP9 were expected to express recombinant DPP8 and DPP9 proteins containing 882 and 892 amino acids, respectively, with additional His6, S and Trx tags in the N-terminus.

2.3. Prokaryotic expression, protein purification and Western blot

Rosetta competent cells transformed with the pET32-a(+)-DPP8 plasmid were cultured in LB medium containing 50 μ g/mL ampicillin at 37 °C. Protein expression was induced by isopropyl β -D-thiogalactopyranoside (IPTG) (1 mmol/L) at 16 °C for 20 h, until the OD₆₀₀ value of the LB medium reached 0.6. The cells were harvested at 6000 rpm for 5 min and incubated in lysozyme buffer (2 mg/mL lysozyme, 100 mmol/L Tris–HCl pH 7.4, 100 mmol/L NaCl) for 40 min on ice. This was followed by centrifugation at 12,000 rpm for 10 min and filtration through a 0.45 μ m filter. The filtrate was purified with a nickel affinity column as follow: the column was equilibrated with binding buffer (20 mmol/L Tris–HCl pH 7.9, 10 mmol/L imidazole, 0.5 mol/L NaCl), and then the supernatant was passed through the column. Subsequently, binding buffer was used to wash the column, followed by elution buffer (20 mmol/L Tris–HCl pH 7.9, 500 mmol/L imidazole, 0.5 mol/L NaCl). All procedures were done at 4 °C. The enzyme activities of the eluted fractions were monitored, and the active fractions were pooled and analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. The expression and purification procedures of DPP9 were the same as DPP8.

2.4. Determination of the optimum concentrations of the substrate and purified recombinant proteins

To determine the optimum concentrations of the substrate and purified recombinant proteins, the assay was performed with different concentrations of DPP8 or DPP9 protein (0–60 ng/mL) and a concentration range of Gly-Pro-*p*-nitroanilide (0–0.5 mmol/L) in HEPES buffer (pH 7.05) in a final volume of 100 μ L. The change in absorbance at 405 nm was kinetically monitored every 10 min for 120 min at 37 °C.

2.5. DPP selectivity evaluation based on DPP8/9 and DPP4 inhibitor screening method

2.5.1. DPP selectivity evaluation based on DPP8/9 inhibition assays

For the practicality and applicability of the inhibition assays, the proven DPP4 inhibition assay was referenced, which indicated the changes of OD values were 0.1–0.2 in 60 min. According to the optimised concentration detection results described above, the final

concentrations of test compounds and Gly-Pro-*p*-nitroanilide were chosen to be 10 $\mu\text{mol/L}$ and 0.2 mmol/L, respectively; and the concentrations of DPP8 and DPP9 were 30 ng/mL and 20 ng/mL, respectively. The change in absorbance at 405 nm was monitored for 60 min at 37 $^{\circ}\text{C}$. Distilled water was used as a negative control. The percentage of inhibition was calculated as shown below.

$$\text{Inhibition (\%)} = (\Delta\text{OD}_{60-0} \text{ of control} - \Delta\text{OD}_{60-0} \text{ of test}) / \Delta\text{OD}_{60-0} \text{ of control} \times 100\%$$

2.5.2. The screening method of DPP4 inhibitor

The test well contained 10 μL compound solution (diluted to the concentration of 10 $\mu\text{mol/L}$) and 50 μL DPP4 solution (2 mU/mL in 50 mmol/L Tris-HCl buffer, pH 8.0) in a 96-well plate. The enzyme reaction was started by addition of 40 μL Gly-Pro-*p*-nitroanilide solution (0.26 mmol/L, in HEPES buffer, pH 7.05). The change in absorbance at 405 nm was monitored for 60 min at 37 $^{\circ}\text{C}$. Compound solution was replaced by the distilled water in the negative control well. The percent of inhibition was calculated as above.

Two different DPP inhibitors, sitagliptin and UAMC00132, were used to confirm the specific inhibition of DPP8/9 and DPP4.

2.6. Statistical analysis

All values were presented as mean \pm S.E.M. The Sigma Plot software was used for data analysis.

3. Results

3.1. Construction of prokaryotic DPP8/9 expression plasmids

Using RT-PCR, we obtained 2649 bp and 2679 bp fragments of DPP8 and DPP9, respectively, with extra primer bases (Fig. 1A). Using the additional restriction sites, the DNA fragments were successfully inserted into the pET32-a(+) (~5,900 bp) plasmid and identified by restriction digestion (Fig. 1B and C) and sequencing.

3.2. Protein expression, purification and identification

The recombinant human DPP8 and DPP9 proteins were successfully expressed in the Rosetta cells induced by IPTG (1 mmol/L) and analysed by SDS-PAGE. The corresponding bands of DPP8

and DPP9 were both at approximately 120 kDa (Fig. 2A and C). The recombinant DPP8 and DPP9 proteins with His₆-tags were purified on a nickel affinity column and then dissolved in the neutral Tris buffer. The soluble, purified target proteins were also approximately 120 kDa (Fig. 2B and D), which was confirmed by Western blot analysis using an anti-His antibody (Fig. 2E and F).

3.3. Determination of the optimum concentrations of DPP8/9 and substrate in a direct enzyme inhibition assay

The recombinant human DPP8 and DPP9 proteins were found to display specific dipeptidyl peptidase activities similar to DPP4, but the enzyme kinetic characteristics were unknown. By monitoring the increasing absorbance rate at 405 nm, concentration-activity experiments of recombinant DPP8/9 were performed. The experiment showed that the reactivity of recombinant DPP9 was higher than DPP8. To determine the reliable and practical reaction conditions that were suitable for the soluble DPP8 and DPP9 activity inhibition assay, the proven DPP4 inhibition assay was referenced, which indicated the changes of OD values were 0.1–0.2 in 60 min. The experiment indicated that the optimum concentrations of the purified recombinant DPP8 and DPP9 proteins were 30 ng/mL and 20 ng/mL for 60 min, respectively, with 0.5 mmol/L substrate (Fig. 3A and B), and the optimum substrate concentration for both DPP8 (30 ng/mL) and DPP9 (20 ng/mL) was 0.2 mmol/L (Fig. 3C and D).

3.4. DPP inhibition selectivity evaluation based on a direct DPP8/9 and DPP4 enzyme inhibition assay

Using the recombinant DPP8/9 activity assay with the above optimised reaction conditions and the DPP4 activity assay method, the effects of two reported DPP inhibitors on DPP8/9 and DPP4 activities were determined. DPP8/9 inhibitor UAMC00132 (10 $\mu\text{mol/L}$) could, while DPP4 inhibitor sitagliptin (10 $\mu\text{mol/L}$) could not, inhibit DPP8 and DPP9 activities. Meanwhile, sitagliptin strongly inhibited DPP4 activity strongly, while UAMC00132 only slightly inhibited DPP4 activity (Fig. 4), which indicated that the selective evaluation method was established to evaluate the DPP8 and DPP9 selectivity of DPP4 inhibitor candidates.

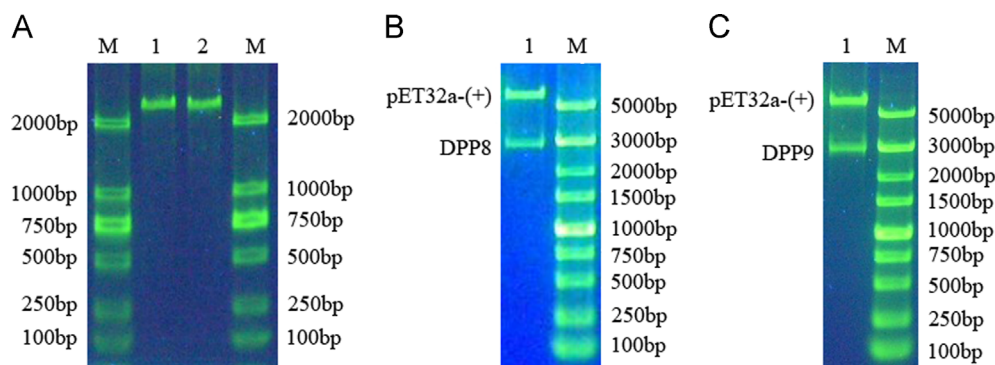


Figure 1 Identification of the recombinant plasmids by restriction digestion. (A) RT-PCR of human DPP8 and DPP9. M: DNA marker; Line 1: DPP8; Line 2: DPP9. (B) Identification of the recombinant pET32-a(+)-DPP8 plasmid. M: DNA marker; Line 1: pET32-a(+)-DPP8 plasmid. (C) Identification of the recombinant pET32-a(+)-DPP9 plasmid. M: DNA marker; Line 1: pET32-a(+)-DPP9 plasmid.

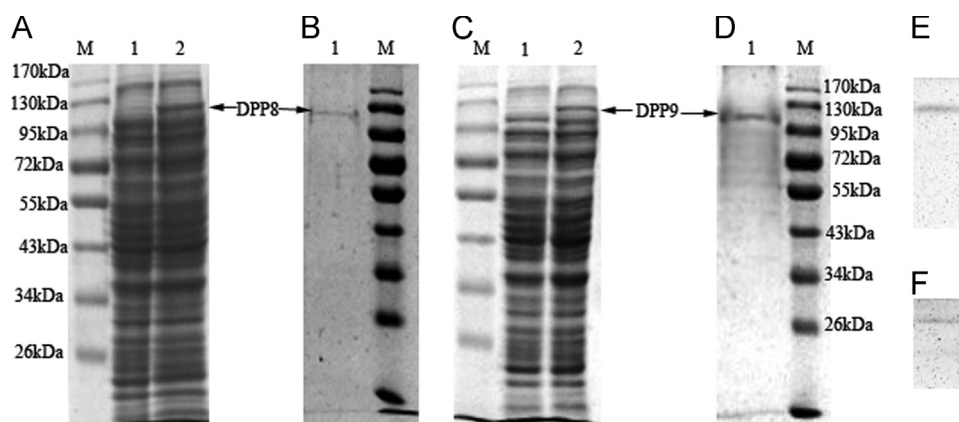


Figure 2 SDS-PAGE and Western blot analyses of recombinant DPP8 and DPP9 proteins. (A) Expression of DPP8 protein. M: protein marker; Line 1: negative control without induction by IPTG; Line 2: the expression of recombinant DPP8 protein induced by IPTG; (B) Purification of DPP8 protein. M: protein marker; Line 1: recombinant DPP8 protein purified using a nickel affinity column. (C) Expression of DPP9 protein. M: protein marker; Line 1: negative control without induction by IPTG; Line 2: the expression of recombinant DPP9 protein induced by IPTG; (D) Purification of DPP9 protein. M: protein marker; Line 1: recombinant DPP9 protein purified using a nickel affinity column. (E) Western blot analysis of purified DPP8 protein using an anti-His antibody. (F) Western blot analysis of purified DPP9 protein using an anti-His antibody. The arrow marks the corresponding recombinant protein at approximately 120 kDa.

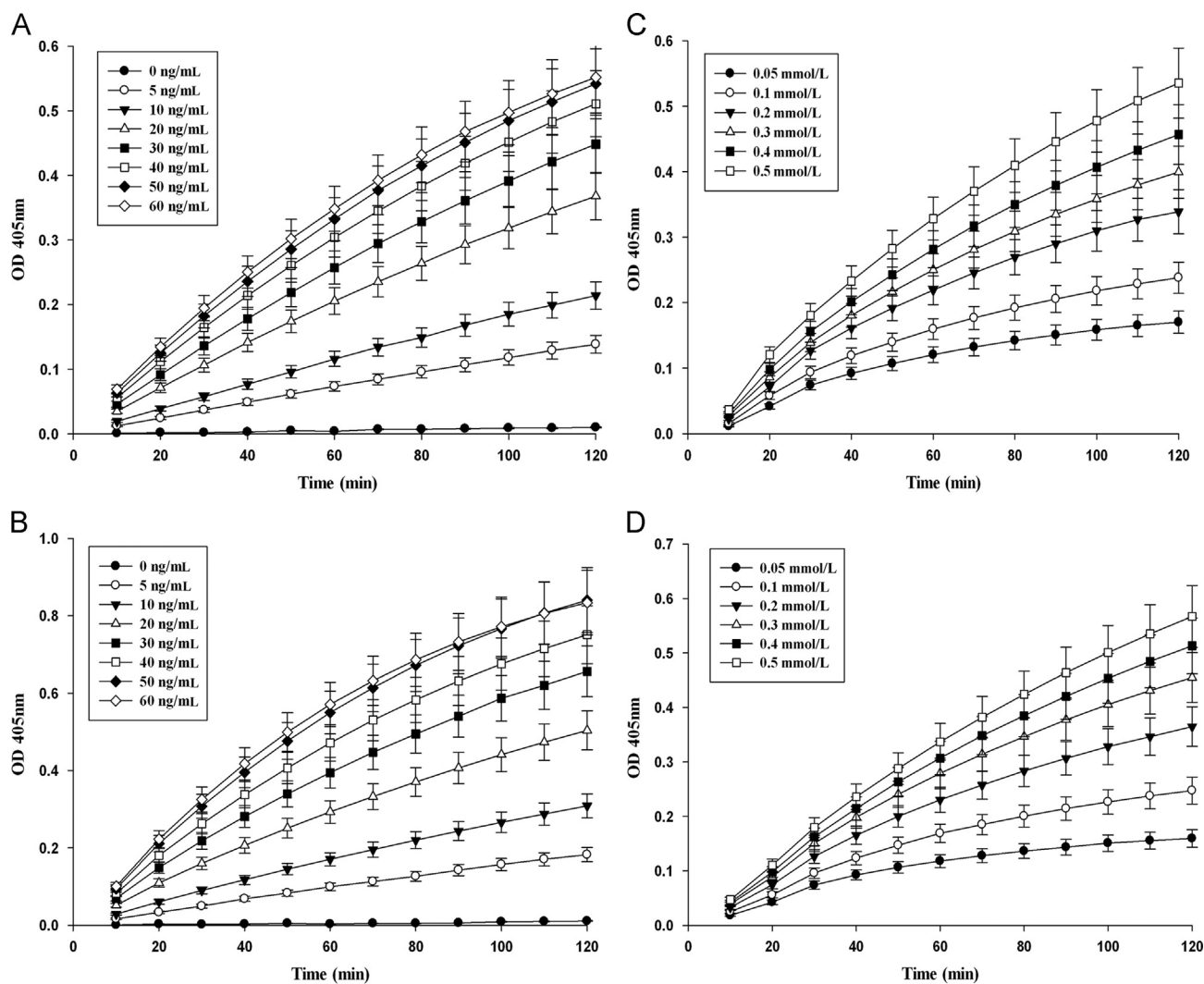


Figure 3 Determination of the optimum concentrations of the purified recombinant DPP8/9 protein and substrate. The purified recombinant DPP8 (A) and DPP9 (B) proteins, with a range of concentrations from 0 to 60 ng/mL and 0.5 mmol/L substrate; The substrate with different concentrations, from 0.05 to 0.5 mmol/L at 30 ng/mL DPP8 (C) and 20 ng/mL DPP9 (D).

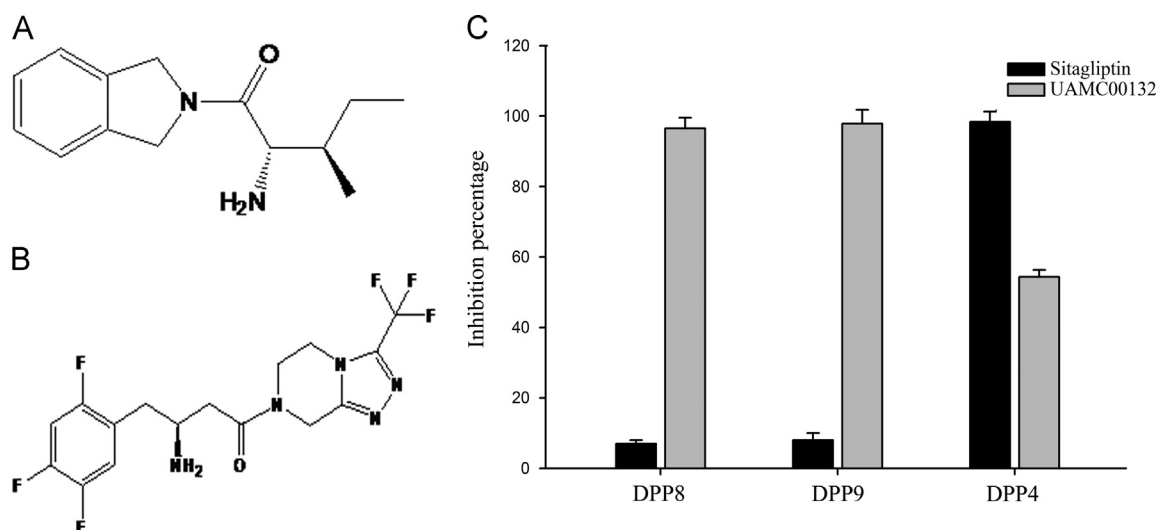


Figure 4 Inhibition of recombinant DPP8/9 and DPP4 activities by UAMC00132 and Sitagliptin. Chemical structure of UAMC00132 (A) and sitagliptin (B). (C) The DPP8/9 and DPP4 inhibitory activities of sitagliptin and UAMC00132. In DPP8/9 inhibition assays, 30 ng/mL purified DPP8 protein or 20 ng/mL purified DPP9 protein and 0.2 mmol/L substrate were used, and in DPP4 inhibition assay, 1 μM DPP4 protein and 0.1 mmol/L substrate were used. The result was presented as the percentage of enzyme activity inhibition.

4. Discussion

The two DPP4 homologues, DPP8 and DPP9, were identified in the years 2000⁴ and 2002⁵. Especially Lankas et al.⁸ found that the inhibition of DPP8 and DPP9 activities could cause severe toxicities. Since then, DPP8 and DPP9 have become popular issues, and a great deal of attentions have been focused on the genes, substrates, inhibitors, structures and functions of these peptidases. In this study, we established a selective evaluation method of DPP4 inhibitors based on the recombinant human DPP8 and DPP9 proteins, which would facilitate and accelerate finding potent and highly selective DPP4 inhibitors.

We used Rosetta cells to express the full length 882 amino acid DPP8 (DPP8_{882aa}) and 892 amino acid DPP9 (DPP9_{892aa}) proteins with additional His₆, S and Trx tags in the N-terminus and with or without another His₆-tag in the C-terminus. In these recombinant variants, we found that only the full length DPP8 and DPP9 proteins without the C-terminus His₆-tag had enzymatic activities, which was consistent with a report by Bjelke⁹. Some reports showed that the purified recombinant DPP8 and DPP9 proteins were monomeric when C-terminus tags were present, but when tags were in the N-terminus, the protein could be dimeric^{4,7,9}. As dimerisation was shown to be essential for the peptidase activity, we considered that the C-terminus His₆-tag of recombinant DPP8/9 may prevent dimerisation and cause a loss of activity. Conversely, other studies have reported active DPP9 with a C-terminus His₆-tag expressing in a eukaryotic system, such as *Pichia pastoris* and *Spodoptera frugiperda* 9 (Sf9) cells, and assumed that the presence of post-translational modifications in mammalian cells may influence the activity of recombinant DPP9^{10–14}. In this study, active recombinant DPP8/9 with an N-terminal His₆-tag, first expressing in a prokaryotic system, showed practical and suitable activity for use in a DPP inhibitor assay.

The Glu–Glu motif in DPP4 (Glu²⁰⁵–Glu²⁰⁶) was also present in DPP8 (Glu²⁵⁶–Glu²⁵⁷) and DPP9 (Glu²⁷⁷–Glu²⁷⁸), which was very important to the peptidase activity and substrate entry, and any mutation in this motif would abolish the peptidase activity^{7,15}.

Some reports also suggested that the α/β hydrolase domains in both the N- and C-terminus of DPP8 and DPP9 proteins were pivotal to the enzymatic activity and to maintain an intact structure and removing these domains would abolish the enzymatic activity⁷. We also expressed the truncated Δ 659–882 amino acid of DPP8 (DPP8 _{Δ 659–882aa}) with additional His₆, S and Trx tags in the N-terminus. We further confirmed that the lack of the Glu–Glu motif and the α/β hydrolase domain of the N-terminus completely abolished enzymatic activity, even though DPP8 _{Δ 659–882aa} contained the catalytic domain.

In summary, we had established a selective evaluation method for DPP4 inhibitor candidates based on the recombinant human DPP8 and DPP9 proteins. This method was highly reproducible and reliable and would provide valuable guidance in the development of promising selective and safe DPP4 inhibitors. We had used the method to evaluate a number of DPP4 inhibitor candidates.

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