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Peptide modulators of alpha-glucosidase

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ABSTRACT

Aims/Introduction: Acute glucose fluctuations during the postprandial period pose great risk for cardiovascular complications and thus represent an important therapeutic approach in type 2 diabetes. In the present study, screening of peptide libraries was used to select peptides with an affinity towards mammalian intestinal alpha-glucosidase as potential leads in antidiabetic agent development.
Materials and Methods: Three phage-displayed peptide libraries were used in independent selections with different elution strategies to isolate target-binding peptides. Selected peptides displayed on phage were tested to compete for an enzyme-binding site with known competitive inhibitors, acarbose and voglibose. The four best performing peptides were synthesized. Their binding to the mammalian alpha-glucosidase and their effect on enzyme activity were evaluated.
Results: Two linear and two cyclic heptapeptides with high affinity towards intestinal alpha-glucosidase were selected. Phage-displayed as well as synthetic peptides bind into or to the vicinity of the active site on the enzyme. Both cyclic peptides inhibited enzyme activity, whereas both linear peptides increased enzyme activity.
Conclusions: Although natural substrates of glycosidase are polysaccharides, in the present study we successfully isolated novel peptide modulators of alpha-glucosidase. Modulatory activity of selected peptides could be further optimized through peptidomimetic design. They represent promising leads for development of efficient alpha-glucosidase inhibitors.

INTRODUCTION

Non-insulin-dependent diabetes (type 2) is characterized by insulin resistance and resultant acute glucose fluctuations during the postprandial period. This triggers oxidative stress and represents greater risk for cardiovascular complications than chronic sustained hyperglycemia, suggesting that postprandial hyperglycemic spikes should be the focus of a therapeutic approach in type 2 diabetes[1,2].

Inhibitors of small intestinal α-glucosidase were shown to improve postprandial hyperglycemia in the 1970s, and were approved as therapeutic agents for diabetes in the 1990s (acarbose)[3]. Despite the fact that they are believed to be less effective in lowering of glycated hemoglobin than sulphonilureas or metformin[4], inhibition of enzymes involved in the digestion of carbohydrates can significantly decrease the postprandial increase of blood glucose level after a mixed carbohydrate diet, and is therefore shown to be essential in preventing the progress of impaired glucose tolerance toward type 2 diabetes[5–8].

In humans, the final phase of starch digestion implicates four small intestinal mucosal α-1,4-exoglucosidases[9,10]. These can be classified as two enzymes with alpha-glucosidase activity: maltase-glucoamylase (MGAM) and sucrase-isomaltase (SI). Each of them has two catalytic domains, an N-terminal (NtMGAM, NtSI) and a C-terminal one (CtMGAM, CtSI). The four catalytic domains are 40–60% identical in amino acid sequence[9,11,12]. Ideally, alpha-glucosidase inhibitors should bind to all four alpha-glycosidase domains in order to inhibit oligosaccharides hydrolysis[11,12].

Studies regarding structural features of alpha-glucosidase inhibitors have shown the characteristic sugar-mimetic structure[13]; however, it is very difficult to prepare a diverse range of sugar derivatives by chemical synthesis or by the isolation of natural products[14]. In contrast, peptides have become increasingly important in the field of drug design[15,16] in the first place because of their high affinity and specificity in interactions with the protein targets, and also because of their reduced immuno-

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genicity and low toxicity profiles. Furthermore, it is relatively easy to prepare a large set of peptides (up to more than 10^9) in a phage display library. Phage display is a very applicable molecular technology used as a high throughput screening approach in drug discovery for the identification of target-specific peptides and proteins for various disease-related targets. The deoxyribonucleic acid (DNA) encoding the random peptide is fused with phage coat protein genes, and the desired protein is expressed on the surface of the phage particle. Methodology allows identification of carbohydrate peptide mimetics[14,17], and was therefore used in the present study to select peptides with an affinity towards mammalian intestinal alpha-glucosidase as potential leads in antidiabetic agent development. Three phage-displayed random peptide libraries were used in independent selections with different elution strategies, which resulted in the discovery of two linear and two cyclic heptapeptides with an affinity towards intestinal alpha-glucosidase. They represent promising leads for development of efficient alpha-glucosidase inhibitors.

MATERIALS AND METHODS

Enzyme Preparation

The methods described by Yoshikawa[18], Mohamed Sham Shihabudeen[1] and Oki[19] for mammalian α-glucosidase purification were combined and slightly modified. Rat intestinal acetone powder (Sigma-Aldrich, St. Louis, MO, USA) was dissolved to 2.5% m/V in ice-cold phosphate buffer pH 7.0 containing ethylenediaminetetraacetic acid and dithiothreitol. The homogenate was sonicated for 15 min at 4°C followed by the addition of 2% Triton X-100 and vigorous vortexing for 20 min. The resultant suspension was centrifuged for 30 min at 12,041 *g*. The supernatant was subjected to sequential 30 and 70% ammonium sulphate precipitations. The resultant pellet was then resuspended in 2 mL of phosphate buffer pH 7.0 and dialyzed overnight against the same buffer. The resultant dialysate was used as a source of mammalian MGAM that was immobilized onto antibody-coated beads.

For the purpose of activity evaluation, the enzyme solution was additionally purified. The dialysate was first concentrated to 500 μL by ultrafiltration and then loaded onto HiPrep Q XL 16/10 column (GE Healthcare, Vienna, Austria), equilibrated with Tris–HCl buffer, pH 8.0. The column was eluted by NaCl linear gradient (0–1 mol/L). The fractions with alpha-glucosidase activity were collected and injected onto Superdex 200 10/300 GL column (GE Healthcare) equilibrated and eluted with phosphate buffer pH 6.8. Fractions of eluate with alpha-glucosidase activity were used in further experiments.

Immobilization of Target Molecule

Protein G-coated magnetic beads (Dynabeads Protein G; Invitrogen, Oslo, Norway) were washed with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (0.05% PBST) and anti-MGAM polyclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were added. After the incubation with mild shaking for 30 min at room temperature, supernatant was removed and the beads were washed three times with 0.05% PBST. Dialyzed enzyme solution was added to coated beads and incubated for 45 min at room temperature with gentle shaking. The remaining solution was then removed and the beads were washed five times with 0.05% PBST.

Affinity Selection

Three different phage-displayed libraries – linear Ph.D.-7™ and Ph.D.-12™, and cyclic Ph.D-C7C™ (New England Biolabs, Frankfurt am Main, Germany) – were used in six independent selections of peptides using immobilized MGAM as the target. Aliquots of 2×10^{11} plaque-forming units from each library in 0.05% PBST were added to MGAM-coated magnetic beads and incubated for 60 min at room temperature. In the subtractive selection step in the second and third round of selection, an aliquot of amplified eluate from a previous round of biopanning was added to anti-MGAM antibody-coated beads. After 30 min of gentle shaking at room temperature, the supernatant was transferred to the enzyme-coated beads and was incubated for 60 min. The beads were washed 10 times with 0.1% PBST, and bound phages were eluted with either 0.5 mmol/L acarbose/0.5 mmol/L voglibose (both Sigma-Aldrich) or acidic elution buffer (20 mmol/L Gly/HCl, pH 2.2). Low pH eluate was immediately neutralized with 1 mol/L Tris/HCl, pH 9.1. Eluates were amplified by infecting *Escherichia coli* ER2738 host cells (New England Biolabs). After 4.5 h of vigorous shaking at 37°C, bacteria were removed by centrifugation. Phage particles were purified by two consecutive precipitations with PEG/NaCl (20% PEG-8000, 2.5 mol/L NaCl) and finally resuspended in PBS. These amplified eluates were titered to determine phage concentration and then used as the input phage for the next selection round. Finally, unamplified eluate from the third round of biopanning was used to infect plated bacterial host cells, and 20–40 resulting plaques were randomly selected. They were individually amplified and purified for further analysis.

Phage Enzyme-Linked Immunosorbent Assay

Microtiter plate wells were coated with 50 μL of anti-MGAM antibodies (5 μg/mL) in PBS and incubated overnight at 4°C. The wells were blocked with 2% bovine serum albumin/PBS for 90 min at room temperature and washed three times with 0.1% PBST. Wells were then incubated with 50 μL of MGAM dialysate for 60 min at room temperature with gentle agitation and washed three times with 0.1% PBST. A separate set of wells was blocked with blocking buffer without target enzyme immobilization to determine background binding levels. A suspension of 5×10^9 plaque-forming units of individual phage clones in 0.05% PBST was added to the wells. After 60 min the wells were washed five times with 0.1% PBST. Bound phages were detected with horseradish peroxidase-conjugated anti-M13 monoclonal antibodies (GE Healthcare). TMB substrate (Thermo Scientific, Waltham, MA, USA) was used for visualization, and the absorbance at 450 nm was measured using a

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microtiter plate reader (Tecan Safire, Grödig, Austria). A total of 21 clones with the highest enzyme to background absorbance ratio were selected for DNA sequencing.

DNA Sequencing

Single-stranded phage DNA was isolated by denaturation of coat proteins with iodide buffer (10 mmol/L Tris/HCl at a pH 8.0, 1 mmol/L ethylenediaminetetraacetic acid, 4 mol/LM NaI) and DNA precipitation with ethanol. Purified DNA was sequenced by GATC Biotech, Konstanz, Germany. The obtained peptide sequences were thoroughly examined using the MimoDB 2.0 database (http://immunet.cn/mimodb) to remove target-unrelated peptides from the pool of potential MGAM binders.

Peptide Synthesis

The selected peptides were synthesized by EZBiolab, Westfield, IN, USA. Where appropriate, peptides were cycled with disulphide bond between external cysteines. In all peptides, C-terminal carboxylate was amidated in order to block the negative charge. The purity of the peptides was over 95%. Identity was determined by mass spectrometry.

Competition Phage Enzyme-Linked Immunosorbent Assay Using Acarbose/Voglibose and Synthetic Peptides

Microtiter plate wells were prepared the same way as for phage enzyme-linked immunosorbent assay (ELISA). Test wells were incubated with 50 μL of 0.5 mmol/L acarbose/0.5 mmol/L voglibose or 0.1 mmol/L solution of synthetic peptides in 0.05% PBST for 30 min with mild shaking at room temperature (to occupy binding sites at the immobilized target). Next, 5×10^9 plaque-forming units of corresponding phages in 0.05% PBST were added into each well and incubated for 45 min. Wells were then washed five times with 0.1% PBST and bound phages were detected as described for phage ELISA.

Evaluation of Enzyme Activity in the Presence of Synthetic Peptides

Alpha-glucosidase activity was measured as described previously[20]. The final eluate (obtained as aforementioned), enriched for alpha-glucosidase activity, was diluted 5000 times in phosphate buffer pH 6.8 and premixed with 0.5 mmol/L acarbose/0.5 mmol/L voglibose or synthetic peptides solution at final concentration of 1.2 mg/mL. 4-Methylumbelliferyl-α-D-glucopyranoside (4-MUG; Sigma-Aldrich) in phosphate buffer was added to the mixture at 0.3 mmol/L as a substrate. The reaction was incubated at 37°C for 120 min and stopped by adding 0.2 mol/L Na_2CO_3. Alpha-glucosidase activity was determined by measuring fluorescence of released 4-methylumbelliferone anion at λex 365 nm and λem 445 nm.

Statistical Analysis

For all tests, the assays were carried out in triplicates. When examining the inhibitory potential of synthetic peptides, the control values (enzyme activity without inhibitor) were measured six times, and all other measurements were carried out three times. The values are represented as mean ± standard deviation. Statistical comparisons were made with the Mann–Whitney *U*-test.

RESULTS

Affinity selections were carried out using three commercially available phage-displayed peptide libraries in order to select alpha-glucosidase-binding peptides capable of modifying enzyme activity. Two selections were carried out with each library, taking either a specific or non-specific elution approach. After the last round of biopanning, 20–40 random clones from each selection protocol were assessed in ELISA assay to determine the binding affinity of the displayed peptides to the target enzyme and background decoy proteins. A total of 21 bacteriophage clones that showed highest selectivity for the target were selected and sequenced. Displayed peptides are listed in Table 1.

Peptide sequences were then examined through the search of the collection of published data and online mimotope databases[21–23] in order to eliminate any known target-unrelated results. From the remaining peptides, six clones with the highest target to the background binding ratio were additionally evaluated. To determine the binding site of the peptides, a competition ELISA with a mixture of known competitive inhibitors, acarbose and voglibose, was carried out. Competitive inhibitors significantly prevented binding of all six phage clones to the immobilized enzyme (Figure 1a), suggesting binding of phage-displayed peptides into or to the vicinity of the active site of the enzyme. Additionally, low binding of isolated phage clones to background proteins (BSA and anti-MGAM antibodies used in the enzyme immobilization step in all selections) was observed.

We further evaluated four phage clones with positive binding to the target, which was defined as measured absorbance ≥0.4 and enzyme to background absorbance ratio ≥4. Binding of phage clones to the enzyme and BSA at increasing concentrations of bacteriophages was tested (Figure 1b). With all four clones, the difference in absorbance increased along with the amount of phage used. This was most evident for C1 and C2, suggesting lower background binding of cyclic peptides obtained with acidic elution approach.

Analysis of Synthetic Peptides

Binding of synthetic peptides (pA1, pA5, pC1 and pC2) to the enzyme was evaluated through competition ELISA assay. As shown in Figure 2a, there is a considerable reduction of binding of bacteriophage clones to the MGAM in all four cases as a result of the previous incubation with a solution of the corresponding synthetic peptide. However, cyclic synthetic peptides prevented binding of corresponding phage clones to a greater extent than linear peptides. Cyclic peptides also show a higher affinity towards MGAM when displayed on the bacteriophage surface, as seen in Figure 1a,b. Synthetic peptides retained their

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Table 1 | Phage-displayed peptides with highest target-to-background binding

Linear heptapeptide library			Cyclic heptapeptide library			Linear dodecapeptide library		
B1	**4x**	**FHGAREM**	**C1**	**2x**	**CTHYGFRGC**	**E1**	**1x**	**RDGSIAMHSMIP**
B2	1x	TTYSRFP	**C2**	**1x**	**CGHHHRDYC**	D1	1x	QALLEGNAKGGN
A1	**1x**	**NEISFHA**				D2	1x	GGTKTHVDFSLK
A2	6x	LPLTPLP						
A3	1x	LPLGHHE						
A4	1x	IGHLSFE						
A5	**1x**	**GHLYDDP**						

Peptides denoted B, C and E were selected using non-specific elution, and peptides denoted A and D were selected using competitive elution. Peptides with sequences in bold have been further analyzed.

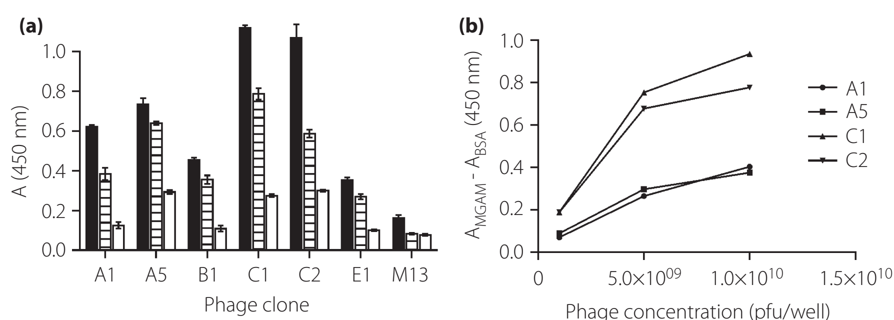

Figure 1 | (a) Binding of selected phage clones to maltase glucoamylase (MGAM; black bars) and to the background (bovine serum albumin [BSA] and anti-MGAM antibody coated wells; white bars). The striped bars show binding of phage clones to MGAM in the presence of a competitive inhibitor (0.5 mmol/L acarbose and 0.5 mmol/L voglibose). M13 phage vector showing no peptide was used as control. (b) Binding specificity of four selected phage clones to MGAM. The graph shown the differences in absorbances measured in MGAM-coated and blank microtiter wells at increasing amounts of phage particles.

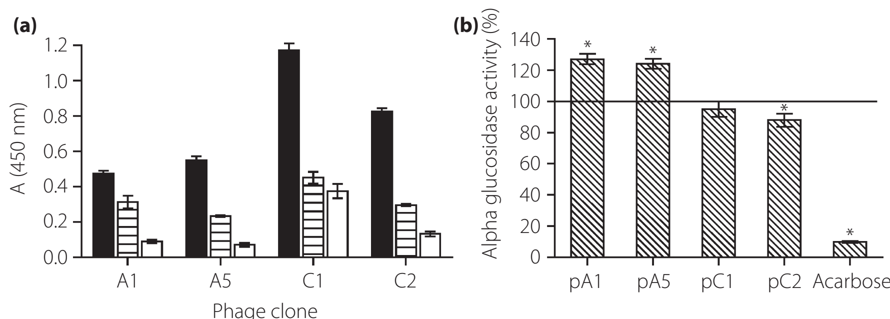

Figure 2 | (a) Binding of phage clones to maltase glucoamylase (MGAM) in the presence of respective synthetic peptide. Black bars represent binding of phage clones alone to the MGAM, whereas striped bars represent binding of same clones to MGAM after preincubation of enzyme with 0.1 mmol/L corresponding synthetic peptide. White bars show binding of phage clones to blank wells. (b) Residual enzyme activity in the presence of synthetic peptides at 1.2 mmol/L concentration. The 100% enzyme activity position is marked with a line. Significant inhibition (12%) was observed with peptide C2. *P = 0.0238, compared with 100% enzyme activity.

ability to bind to the same binding site, as the corresponding phages.

Finally, peptides were tested for their effect on enzyme activity. At 1.2 mmol/L concentration of synthetic peptides, linear peptides slightly elevated the activity of the enzyme (P = 0.0238). In contrast, as seen in Figure 2b, cyclic peptides inhibited alpha-glucosidase. There is no consensus motif among CTHYGFRGC and CGHHHRDYC other than a disulfide bridge between external cysteines, and whereas the inhibition by CTHYGFRGC was not significant, the peptide CGHHHRDYC inhibited alpha-glucosidase activity of rat intestinal MGAM by 12.02 ± 4.23% (P = 0.0238).

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DISCUSSION

Many studies on type 2 diabetes prevention in recent years have been focusing on the inhibition of alpha-glucosidase activity. Natural and synthetic inhibitors of alpha-glucosidase have been identified[11,24–27], but their inhibitory effect depended greatly to the enzyme's origin. Major differences have been observed between yeast and mammalian alpha-glucosidase activity[28], whereas in a recent report the results of hydrolyzing activity of small intestinal disaccharidases from rats showed that experiments evaluating inhibitors on rat intestinal enzymes could substitute for evaluations using human enzymes[29]. The aim of the present research was to obtain results applicable to human intestinal enzymes. Thus, the enzyme origin was prioritized and a crude isolate of rat intestinal membrane proteins, enriched for alpha-glucosidase, was used in the target immobilization step even though ideally no decoy proteins should be present in the coating solution in order to successfully select target-specific ligands from phage-display libraries. Nevertheless, successful panning strategies have been reported before where the whole sera or protein-rich isolates were used as a source of target proteins[30,31].

Furthermore, tethering of the target with specific antibodies in the target immobilization step of panning allowed for specific isolation of the target enzyme from the coating solution. Such an approach also minimized the enzyme's conformational changes and thus supported the retention of enzyme's biological activity throughout the selection. The target protein was properly orientated through entire panning, which is essential for successful selection. Utilization of paramagnetic beads offered increased surface area for binding as well as increased ease and thoroughness of washing.

It is vital to favor a high yield of the fittest clones in the first round of selection, as each clone is represented by only a few copies in the initial library[32]. Thus, a greater amount of target protein was used in the first round than in subsequent rounds of selection. Also, a subtractive selection step was carried out in all but the first round in order to remove phage clones with affinities towards anti-MGAM antibodies or other decoy proteins, and hence to achieve specificity of phage-displayed peptides towards the immobilized target protein.

Judging from ELISA signals, phages displaying cyclic peptides showed a greater affinity towards the target compared with phages displaying linear peptides (Figure 1b). This is in accordance with previously published results[33]. It is generally believed that reduced flexibility of constrained peptides increases their potential for high-affinity binding. The constrained nature of cyclic peptides causes them to adopt a tertiary structure, enabling mimicry of conformational epitopes. Cyclized peptides thus present a more uniform structure than linear peptides. Furthermore, they are less prone to enzymatic degradation than linear peptides[27,34]. Peptide rigidity aside, phage clones isolated with a non-specific elution approach (C1 and C2) appeared to have a higher affinity towards the target than those obtained with specific elution with acarbose/voglibose. Stronger target–peptide interactions have been broken by low pH buffer than by competitive elution buffer.

As ligands are enriched in phage display only according to their affinity towards target protein binding, their biological activity cannot be anticipated. In the present study, cyclic peptides inhibited target enzyme, whereas the linear peptides increased alpha-glucosidase activity of rat intestinal MGAM. There are four catalytic domains of intestinal alpha-glucosidase. The extract used in enzyme activity assay has been enriched for MGAM without differentiation for ntMGAM or ctMGAM, and it is realistic to anticipate both subunits of SI being present as well. It has been shown that alpha-glucosidase subunits differ in their substrate specificities and could be differentially inhibited[9,10,35,36]. In humans, MGAM is more active than SI, but SI is approximately 20-fold more abundant[10]. Linear peptides, pA1 and pA5, could bind to domains with lower affinity for the substrate, which is in turn redirected to domains that have higher catalytical activity. Consequently, the activity of the alpha-glucosidase appears to be elevated.

Cyclic peptide pC2 inhibited alpha-glucosidase by 12%. As the target enzyme is a glycosidase, its natural substrate is polysaccharide. Carbohydrate-mimetic peptides that bind to enzymes have been discovered previously; however, anticarbohydrate antibodies are mainly used as targets in phage display[14] contrary to the approach used in the present study. It has been shown previously that peptides binding specifically to carbohydrate-binding proteins might not necessarily show the corresponding biological activity[37]. Thus, it is noteworthy that at the concentration of 1.2 mmol/L, cyclic peptide CGHHHRDYC significantly inhibits alpha-glucosidase.

The inhibition might not be extensive, nevertheless the selected and characterized lead peptide is yet to undergo multiple structural changes in the forthcoming process of peptidomimetic design. This is a long-established practice in the development of drugs from the peptide scaffold, as it is difficult to use peptides as oral agents. Low enzyme resistance together with low cellular uptake, high rate of hepatic and renal clearance, and high biodegradability all add up to low oral bioavailability and are most important limitations of peptides as therapeutic agents. Through peptidomimetic design, small molecules with the ability to mimic the structure or action of the raw lead peptides, but with better physicochemical properties and pharmacokinetic profiles, are developed (by substituting the natural amino acids by unusual amino acids like [D-] amino acid, N-methyl-alpha-amino acid, beta-amino acid, by replacing amide bond between two amino acids, by blocking N- or C-termini)[38]. A recent example of a successful peptidomimetic-based inhibitor used for the improvement of glycemic control in the therapy of diabetes is teneglíptin, a novel dipeptidyl peptidase-4 inhibitor, approved for use in Japan in 2013, and developed on the basis of endogenic peptide substrates of DPP-4[39,40].

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To summarize, the present study investigated the potential inhibitory effect of the peptides isolated from the phage display library on alpha-glucosidase. Cyclic peptide CGHHHRDYC showed the highest inhibitory activity. As the first milestone in designing a clinically important antidiabetic agent, it represents a peptide lead to be tailored through peptidomimetic design with an aim of improved pharmacokinetic and pharmacodynamic properties.

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DISCLOSURE

The authors declare no conflict of interest.

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