Structure – function analysis of peptide analogs of SQSPA with respect to α-glucosidase and α-amylase inhibition

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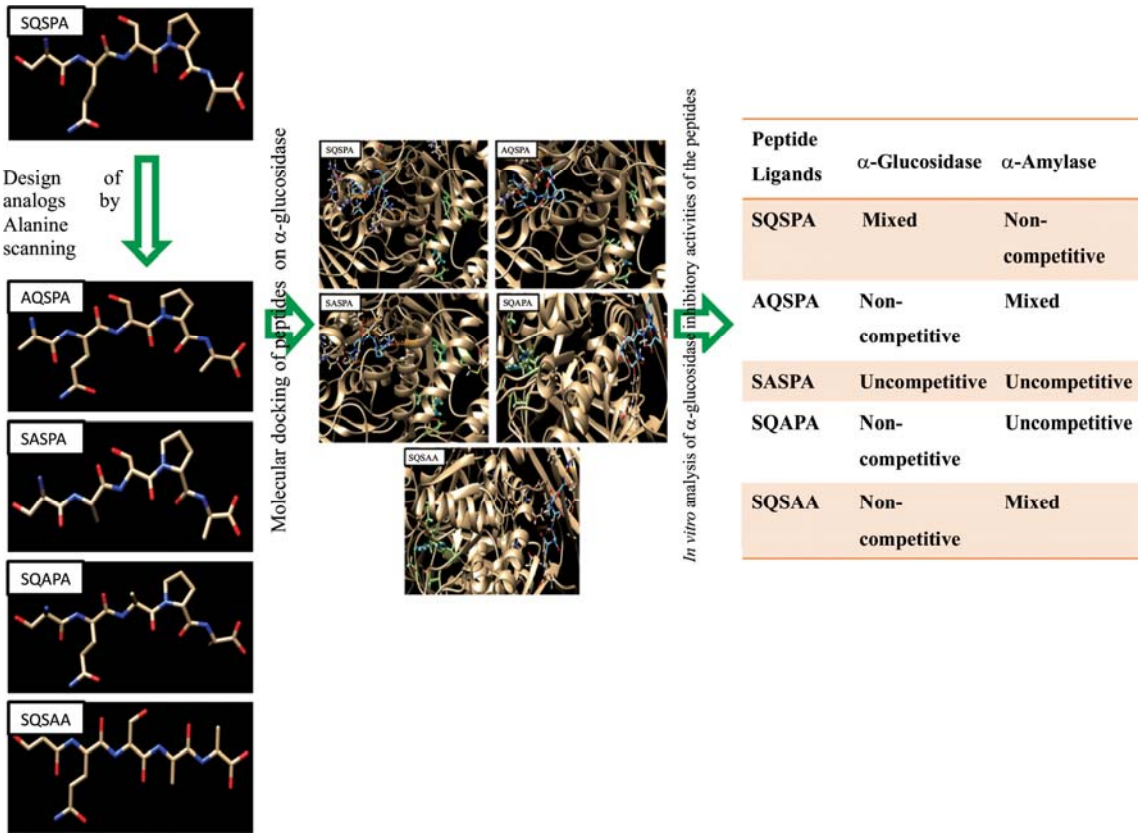
Graphical Abstract



Peptide Ligands	α-Glucosidase	α-Amylase
SQSPA	Mixed	Non-competitive
AQSPA	Non-competitive	Mixed
SASPA	Uncompetitive	Uncompetitive
SQAPA	Non-competitive	Uncompetitive
SQSAA	Non-competitive	Mixed

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Short running title: Alanine scanning of SQSPA for α-glucosidase and α-amylase inhibition

Abstract

Background: Peptide-based therapeutics offer a unique avenue for the development of novel agents for the treatment of diabetes mellitus including α-glucosidase inhibitors. The peptide, SQSPA, was reported to possess to α-glucosidase inhibitory activity in addition to resistance to the gastrointestinal tract (GIT) digestion.

Method: In this study, the *in silico* and *in vitro* structure-activity analyses of the peptide was conducted using alanine scanning to identify key amino acid residues.

Results: The alanine scanning led to four analogs viz; AQSPA, SASPA, SQAPA and SQSAA which were GIT stable. Initially, the peptides were subjected to molecular docking on human α-glucosidase and α-amylase where the binding affinities to the enzymes were in the order; AQSPA>SASPA>SQSPA>SQAPA> SQSAA and AQSPA>SQSAA>SASPA>SQSPA> SQAPA, respectively. Hydrogen bonds were important for the binding of all peptides but SASPA and AQSPA had the highest hydrogen bonds interactions with the α-glucosidase and α-amylase, respectively. *In vitro* analysis revealed that the α-glucosidase and α-amylase inhibitory activities of the peptides were in the order AQSPA>SQSPA>SQAPA>SASPA>SQSAA and AQSPA>SASPA>SQAPA>SQSPA>SQSAA, respectively. Using inhibition kinetics, SQSPA was a mixed inhibitor of α-glucosidase while AQSPA, SQAPA and SQSAA showed non-competitive inhibition. For α-amylase inhibition, SQSPA was a non-competitive inhibitor while AQSPA and SQSAA were mixed inhibitors; SASPA and SQAPA showed uncompetitive inhibition.

Conclusion: The results indicated that P4 and Q2 are important requirements for the α-glucosidase and α-amylase inhibitory activities of the parent peptide, SQSPA. Furthermore, alanine scanning has led to the design of a novel α-glucosidase inhibitory peptide, AQSPA, with increased activities.

Key words: Alanine scanning, diabetes mellitus, disaccharidase, peptide-based therapeutics, structure-activity relationship

1. INTRODUCTION

Over the last decade, peptide-based therapeutics have gained renewed research attention by the biopharmaceutical industries and the scientific community as lead agents in the drug discovery process [1]. This is largely due to their large structural diversity, remarkable efficacy, low toxicity profiles and exquisite selectivity [2, 3]. Moreover, there is a rapid development in strategies to overcome some of the known therapeutic limitations of the peptides such as poor proteolytic stability and low bioavailability due to rapid renal clearance [3, 4]. Currently, there are 60 – 70 approved peptide-based drugs with 100 – 200 in clinical trials and 400 – 600 in preclinical studies covering a wide range of therapeutic applications [3-5]. Indeed, peptide-based therapeutics attracted a market of approximately USD 20 billion from drugs such as Fuseon (HIV), Captopril (hypertension), Zoladex (prostate cancer), Sandostatin – octreotide (acromegaly, endocrine tumors) and Copraxone (sclerosis) [6]. In spite of these developments, candidate peptides against diabetes mellitus and specifically, type 2 diabetes (T2DM) are still in the very early stages of drug discovery which emphasizes the need for more research attention on the development of antidiabetic peptides. This is especially because the prevalence of diabetes mellitus continues to rise globally with current estimation of 451 million people affected by this disease which is expected to rise to 693 million by 2045 [7]. Furthermore, the current global healthcare expenditure for people with diabetes was estimated to be USD 850 billion [7].

The major therapeutic goal in the management of diabetes mellitus is to maintain favourable glycemic control by reducing hyperglycemia and restoring blood glucose levels to near normal. An important therapeutic strategy for the control of the hyperglycemia in T2DM patients is through the inhibition of carbohydrate hydrolysing enzymes such as α-glucosidase and α-amylase [8]. Moreover, the guidelines of the American Diabetes Association and the European Association for the Study of Diabetes have recommended the use of these inhibitors as first-line antidiabetic agents or in combination with other anti-hyperglycemic drugs [8]. However, the clinically available α-glucosidase inhibitors suffer a number of therapeutic setbacks such as flatulence and gastrointestinal (GIT) disturbances which consequently suggests the increasing need for the development of candidate drugs for future evaluation [9]. Therefore, based on the above, our research effort is focused on the identification of peptides with therapeutic potential as α-glucosidase inhibitors [10, 11].

Peptide scanning is an experimental strategy that provides information on the structure-activity relationship of peptides and allows systematic approach for the development of peptide-based therapeutics [6]. It involves systematic modification of each amino acid of a peptide to assess its contribution to the biological activity. Indeed, the identification of critical amino acid(s) required for the biological activity of a peptide is a vital requirement at the early stage of peptide development [6]. Additionally, peptide scanning techniques allow the rational design of peptide analogs for therapeutic purposes [6]. Among the different techniques of peptide scanning, alanine scanning has been the most commonly used to investigate peptides, because the convenient substitution of a residue side chain by a methyl group provides an effective strategy to identify the side chains responsible for binding and pharmacological activity [12]. Consequently, alanine scanning has been extensively used to gain insights into structure-activity relationship of a number of bioactive peptides such as myxinidin [13], feleucin-K3 [14], interleukin-15 peptide antagonist P8 [15] amongst others. Apart from very few reports [16], the application of alanine scanning strategy has not been exploited in the investigation of antidiabetic peptides.

In our previous article, we observed that, so far, a total of 43 fully sequenced peptides with α-glucosidase inhibitory activity have been reported in the literature [10] and *in silico* simulated GIT digestion revealed that all the highly active peptides were gastrointestinally unstable except SQSPA [10], indicating that this peptide has the best therapeutic potential. In the present study, alanine scanning strategy was used to design the peptide analogs of SQSPA and determine the structure-activity relationship of the peptide with respect to α-glucosidase and α-amylase inhibition.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Yeast α-glucosidase, porcine pancreatic amylase, p-nitrophenyl-α-D-glucopyranoside (pNPG), acarbose, starch, maltose and dinitrosalicylic acid (DNS) were obtained from Sigma-Aldrich (Johannesburg, South Africa). The peptides analogs of SQSPA were procured from GenScript (New Jersey, USA).

2.2 Design of the SQSPA analogs

To design the peptide analogs of SQSPA, alanine was substituted in the position of each of the amino acid which led to four analogs (AQSPA, SASPA, SQAPA and SQSAA). Details of the peptide sequences along with the physicochemical parameters of these peptides are presented in Table 1.

TABLE 1 Design of SQSPA analogs and their physicochemical parameters

Peptide sequence	Molecular weight	Net Charge	Isoelectric point	Hydrophobicity (%)	GIT stability
SQSPA	488.50	0	5.24	40	Undigested
AQSPA	472.50	0	5.57	60	Undigested
S**A**SPA	431.45	0	5.24	60	Undigested
SQ**A**PA	472.50	0	5.24	60	Undigested
SQS**A**A	462.46	0	5.24	40	Undigested

Molecular weight and isoelectric point were computed using Expasy Bioinformatics Resource portal (http://web.expasy.org/protparam/). Hydrophobicity was calculated from peptide hydrophobicity/hydrophilicity analysis program at (http://peptide2.com/N_peptide_hydrophobicity_hydrophilicity.php)

Simulated gastrointestinal stability was determined with a combination of chymotrypsin, trypsin and pepsin in the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep)

2.3 *In silico* simulated gastrointestinal digestion of the designed peptides

In addition to the potency, an important feature of SQSPA was GIT stability and therefore, the SQSPA analogs were also subjected *in silico* simulated GIT digestion to determine their potential survival in an *in vivo* environment. The simulated digestion was performed using the GIT digestion enzymes of the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep). The "Enzyme(s) action" application in the BIOPEP database was used for simultaneous digestion of the peptides with a combination of digestive enzymes in the GIT; chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4) and pepsin (pH 1.3) to mimic the *in vivo* digestion.

2.4 Acquisition and preparation of receptors and ligands for molecular docking analysis

Prior to the *in vitro* experiments to determine structure-activity relationship of SQSPA analogs with respect to α-glucosidase and α-amylase inhibition, molecular docking analysis was initially used to assess the structure-activity relationship of the peptides in terms of the binding affinity and mode of interaction towards both enzymes. The 3D crystal structures of the N–terminal of human intestinal α-glucosidase (PDB ID 3L4Y resolved to 1.80Å by x-ray

diffraction) and human pancreatic α-amylase (PDB ID, 4GQR, resolved to 1.20Å by x-ray diffraction) were retrieved in PDB format from the protein data bank (www.rcsb.org). Thereafter, UCSF Chimera package version 1.11.2 (www.cgl.ucsf.edu/chimera/) [17] was used to remove the co-crystallized ligands and water molecules from each of the protein structure. Subsequently, the dock prep tool of the Chimera software [17] was used to prepare the two proteins for the molecular docking. All default parameters for the dock prep tool in Chimera were used. For the peptide ligands, the 3D structures were drawn with ACD/ChemSketch software (https://www.acdlabs.com/resources/freeware/chemsketch/) and retrieved as Mol files. These peptide ligands were then opened in the Chimera software and subjected to the dock prep tool as earlier described. The structures of the receptors and ligands were retrieved as PDB format after the dock prep.

2.5 Molecular docking analysis with Autodock Vina

The PDB prepared versions of the two proteins and the peptide ligands were opened in Chimera and subjected to the Autodock Vina tool [18] in the same software. The grid sizes (xyz points) were set at 59.78 x 57.72 x 53.91 and 41.46 x 45.35 x 347.23 for α-glucosidase and α-amylase, respectively, while the grid centers were designated at dimensions (x, y and z) 10.48, -7.04 and -19.76 for α-glucosidase and 9.40, 30.41 and 214.67 for α-amylase. Other parameters of Autodock Vina tool in Chimera were left as default. AutoDock Vina employs iterated local search global optimizer and all output files were saved in pdbqt format. After successful docking in Vina, the minimum binding free energy for each of the peptides was recorded and the docking pose was extracted and aligned with the receptor structure for further analysis of hydrogen bond interactions.

2.6 Peptide synthesis and preparation

The five peptides SQSPA, AQSPA, SASPA, SQAPA and SQSAA were synthesized by Flexpeptide™ technology by GenScript. The purity and amino acid analysis of the peptides were determined by the manufacturer using reverse phase high performance liquid chromatography and mass spectrometry. Stock peptide solutions were prepared in sterile deionized double distilled water.

2.7 Assay for α-glucosidase inhibitory activity of SQSPA analogs

The α-glucosidase inhibitory activity was assayed using a previously described method [19] with slight modifications. A volume of 50 µL of each peptide or acarbose at a final concentration range of 62.5 -500 µM was incubated with 25 µL of 0.5 U/mL α-glucosidase solution in 100 mM phosphate buffer (pH 6.8) at 37 ^{o}C for 60 min. Subsequently, 25 µL of pNPG solution (5 mM) in a similar buffer was added and the mixture was further incubated at 37 ^{o}C for 30 min. The absorbance of the released *p*-nitrophenol was measured at 405 nm using a Spectramax paradigm multi-mode microplate reader (Molecular Devices LLC, USA) and the inhibitory activity was expressed as percentage of a control sample without the inhibitors. The α-glucosidase inhibitory activity of the peptides was calculated by using the following formula:

$$\alpha - \text{Glucosidase inhibitory activity } (\%) = \left(1 - \frac{\text{As}}{\text{Ac}}\right) \text{x } 100$$

where As and Ac are absorbance of sample and absorbance of control respectively. The concentrations of the peptides resulting in 50% inhibition of enzyme activity (IC_{50} values) were determined using the straight line equations of the percentage inhibitory activity against the respective logarithm of peptide concentrations [20].

2.8 Assay for α-amylase inhibitory activity of SQSPA analogs

The α-amylase inhibitory activity was assayed using a previously described method [19] with slight modifications. Briefly, 50 µL of each peptide or acarbose at a final concentration range of 62.5 – 500 µM was incubated with 25 µL of 2 U/mL pancreatic α-amylase solution in 100 mM phosphate buffer (pH 6.8) at 37 ^{o}C for 60 min. Thereafter, a volume of 25 µL of 1 g/100 mL starch dissolved in a similar buffer was added to the reaction mixture and incubated at 37 ^{o}C for 60 min. This was followed by the addition of 100 µL of DNS color reagent and the solution was incubated in boiling water for 10 min. The absorbance of the resulting mixture was measured at 540 nm with a Spectramax paradigm multi-mode microplate reader (Molecular Devices LLC, USA) and the inhibitory activity expressed as percentage of a control sample without the inhibitors. The α-amylase inhibitory activity of the peptides was calculated by using the following formula:

$$\alpha - \text{Amylase inhibitory activity } (\%) = \left(1 - \frac{\text{As}}{\text{Ac}}\right) \text{x } 100$$

where As and Ac are absorbance of sample and absorbance of control respectively. The concentrations of the peptides resulting in 50% inhibition of enzyme activity (IC_{50} values) were determined using the straight line equations of the percentage inhibitory activity against the respective logarithm of peptide concentrations [20].

2.9 Mechanism of α-glucosidase and α-amylase inhibition

The peptides were subjected to enzyme inhibition kinetic experiments to determine the type of inhibition exerted on the α-glucosidase and α-amylase. The experiment was performed according to the protocols as described above at two fixed concentrations of the peptides (250 and 500 μM) which are less than $1/10^{th}$ of the K_M value for the enzymes. Moreover, the concentration of substrates was also varied to be 0.625 - 5 mM of *p*NPG and 0.125 - 1 g/100 mL of starch for the α-glucosidase and α-amylase inhibition assays, respectively. The initial velocity data obtained were used to construct Lineweaver-Burk plots to determine the type of inhibition of the peptides against both enzymes.

2.10 Statistical analysis

Values are presented as mean ± SD of two independent experiments done in triplicates and the data were analyzed by using a statistical software package (SPSS for Windows, version 18, IBM Corporation, NY, USA) using Tukey's-HSD multiple range *post-hoc* test. Values were considered significantly different at $p < 0.05$.

3. RESULTS

The sequence and other physicochemical parameters of SQSPA and its analogs are summarized in Table 1. Compared to the parent peptide SQSPA, the alanine substitution resulted in lower molecular weight peptide analogs but did not affect the isoelectric point (5.24) except in the case of substitution at position 1 (AQSPA) where the isoelectric point was increased to 5.57. Interestingly, all the peptide analogs were predicted to be stable following the digestion with a combination of pepsin, chymotrypsin and trypsin (Table 1).

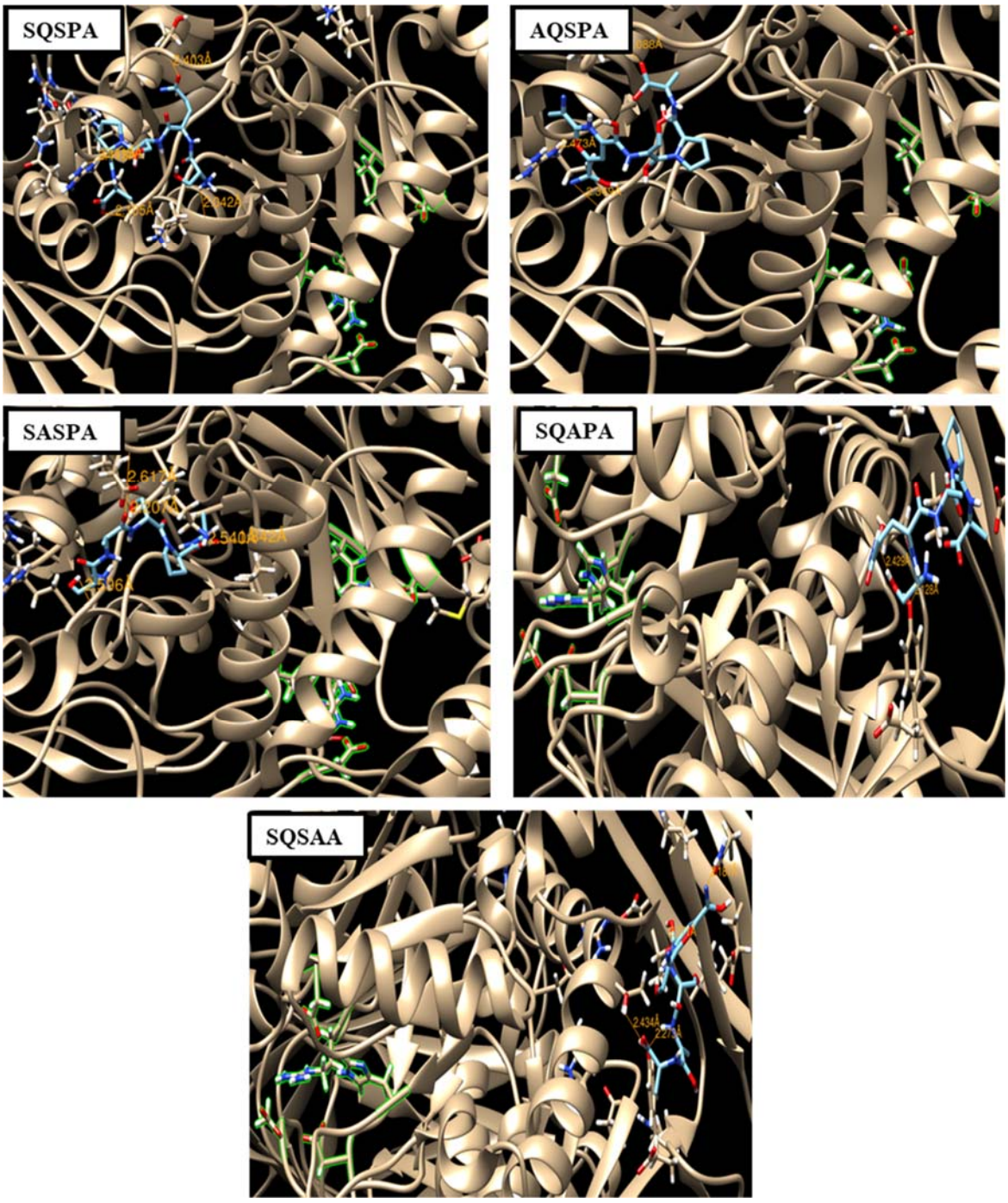

FIGURE 1 Molecular docking pose of SQSPA analogs on human α-glucosidase. The orange line represents the hydrogen bonds with the respective bond distance while the green area indicated the active site residues. The binding site has been zoomed out for each peptide- α-glucosidase interaction and presented.

TABLE 2 Binding free energy (kcal/mol) and other docking results of SQSPA analogs with human α-glucosidase

Peptide ligand	Binding energy (kcal/mol)	Number of hydrogen bonds	Interacting residue of the peptide	Interacting residue of α-glucosidase	Hydrogen bond distance (Å)
SQSPA	-7.0	5	A5	R283	2.105
			P4	R283	2.415
			P4	R283	2.413
			Q2	T778	2.403
			S1	K534	2.042
AQSPA	-7.3	3	Q2	R283	2.349
			A1	R283	2.473
			A5	V779	2.088
S**A**SPA	-7.2	5	S3	L286	2.207
			A5	I523	1.842
			P4	K776	2.540
			A2	D777	2.617
			S1	R283	2.596
SQ**A**PA	-6.7	2	S1	T632	2.429
			S1	Y703	2.128
SQS**A**A	-6.5	4	A5	T632	2.434
			A4	N814	2.187
			Q2	Y703	2.273
			Q2	T737	2.171

The SQSPA analogs were initially subjected to molecular docking studies and all the peptides were found to bind to the α-glucosidase (Fig. 1) with alanine substitution at position 1 (AQSPA) demonstrating the lowest binding free energy (Table 1). Overall, the binding affinity was in the order AQSPA > SASPA > SQSPA > SQAPA > SQSAA indicating that the alanine substitution at positions 1 and 2 increased the binding affinity of the parent peptide SQSPA but the substitution at positions 3 and 4 lowered the binding affinity (Table 2). Although all the peptides did not bind at the active site of the α-glucosidase, SQSPA, AQSPA and SASPA had a similar binding site while SQAPA and SQSAA bind at another distinct site on the enzyme (Fig. 1). With respect to hydrogen bond interactions, the alanine substitution reduced the number of hydrogen bonds between the parent peptide and the enzyme except the substitution at position 2. Hence, SQSPA and SASPA had the highest number (5) of hydrogen bond interactions with the α-glucosidase (Table 2). The positions 1 and 2 were the most hydrogen bonded sites in the peptides and appeared to be the critical

positions for the hydrogen bond formation with the peptide ligands. This is because the hydrogen bond interactions were still observed when the S1 and Q2 (SQSPA) were substituted with alanine to generate AQSPA and SASPA respectively (Table 2). For the α-glucosidase, R283 was involved in the hydrogen bond formation than any amino acid at the binding site of SQSPA, AQSPA and SASPA whilst T632 and Y703 seem to be the vital amino acids for the hydrogen bond interaction with SQAPA and SQSAA.

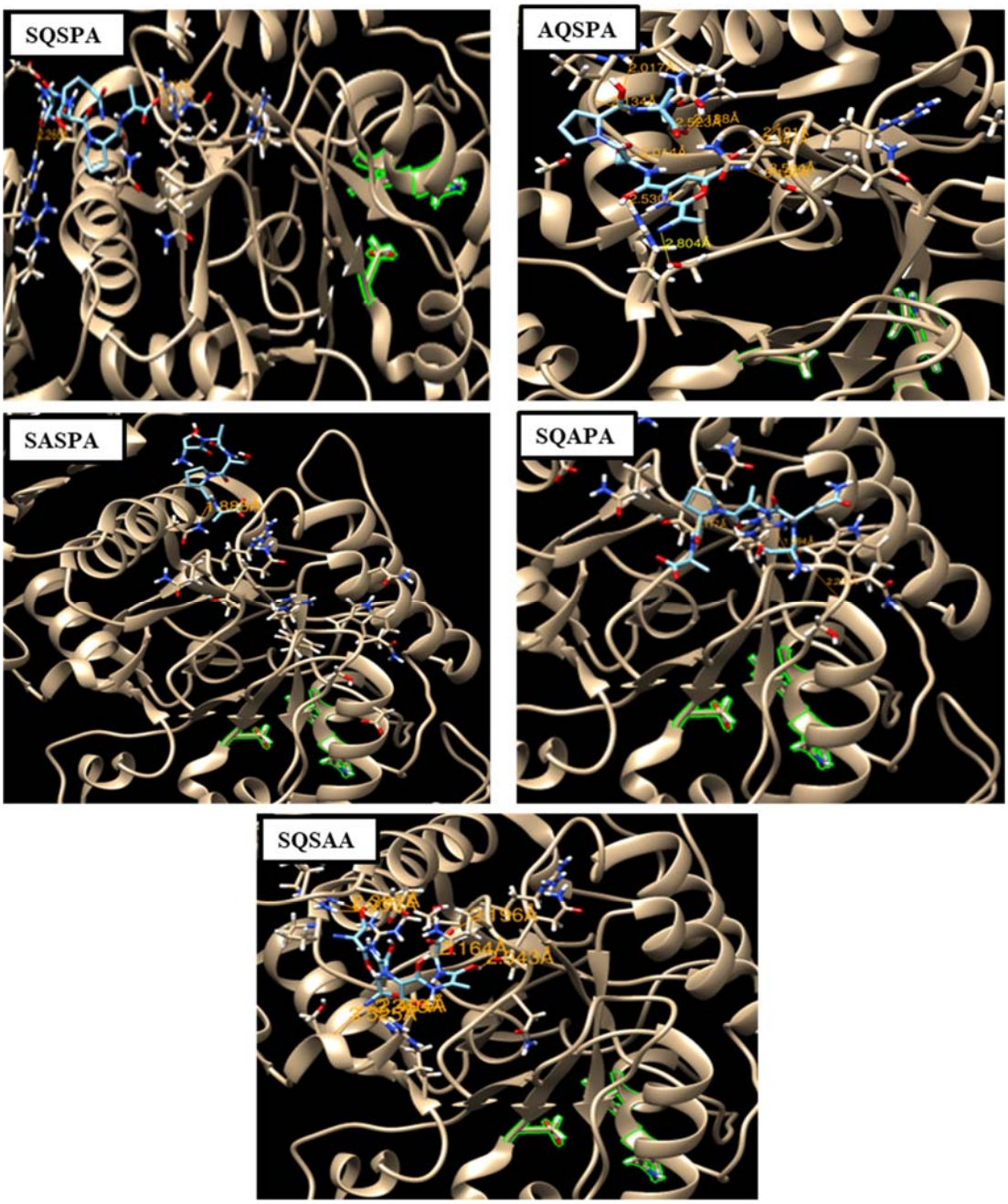

FIGURE 2 Molecular docking pose of SQSPA analogs on human α-amylase. The orange line represents the hydrogen bonds with the respective bond distance while the green area indicated the active site residues. The binding site has been zoomed out for each peptide- α-glucosidase interaction and presented.

TABLE 3 Binding free energy (kcal/mol) and other docking results of SQSPA analogs with human α-amylase

Peptide ligand	Binding energy (kcal/mol)	Number of hydrogen bonds	Interacting residue of the peptide	Interacting residue of α-amylase	Hydrogen bond distance (Å)
SQSPA	-5.7	3	A5	R10	2.281
			A5	R10	2.047
			S1	H491	2.265
AQSPA	-7.8	11	Q2	R252	2.530
			A5	R398	2.188
			A5	R398	2.523
			P4	G403	2.017
			A1	S3	2.804
			Q2	T6	3.156
			Q2	Q7	3.584
			Q2	R10	3.341
			A5	P332	2.134
			Q2	R10	2.191
			S3	G334	2.014
S**A**SPA	-5.8	1	P4	N399	1.888
SQ**A**PA	-5.2	3	S1	S219	2.261
			A3	W221	1.994
			A5	T6	2.177
SQS**A**A	-6.2	8	A5	G9	2.196
			Q2	R252	2.363
			Q2	R252	2.404
			S1	R421	2.387
			S1	R421	1.933
			Q2	S289	3.525
			A4	Q8	2.872
			A4	T6	2.892

α-Glucosidase inhibition is closely related with the corresponding inhibitory effect on α-amylase and therefore, the ability of the peptides to also bind human α-amylase was initially investigated using the molecular docking (Fig. 2). It was observed that all the peptides bind to the α-amylase at a site distant from the active site (Fig. 2) but contrary to the binding affinities with α-glucosidase, alanine substitution at positions 1, 2 and 4 was found to increase the binding affinity of the SQSPA towards the α-amylase. However, the alanine substitution at position 1 (AQSPA) showed the highest binding affinity among all the peptide ligands (Table 3). Moreover, the AQSPA also had the highest number of hydrogen bond

interactions (11) followed by SQSAA with 8 hydrogen bonds (substitution at position 4) while Q2 was important for the hydrogen bond formation. This is evident because alanine substitution at the second position (SASPA) resulted in a single hydrogen bond only (Table 3). The binding residues of the peptide ligands involved in the hydrogen bonds with the α-amylase were highly varied while the bond distance was in the range 1.888 – 3.584 Å.

TABLE 4 IC_{50} values for α-glucosidase and α-amylase inhibition by SQSPA derivatives and acarbose

Peptide/Control	IC_{50} (mM)		Ratio of IC_{50} values of α-glucosidase to α-amylase inhibition
	α-Glucosidase	α-Amylase	
SQSPA	3.09 ± 0.29^{c}	10.30 ± 0.73^{c}	1:3.33
AQSPA	2.42 ± 0.30^{b}	5.35 ± 0.39^{a}	1:2.21
S**A**SPA	6.86 ± 0.93^{e}	7.92 ± 0.36^{b}	1:1.15
SQ**A**PA	4.04 ± 0.34^{d}	8.68 ± 0.39^{b}	1:2.14
SQS**A**A	8.54 ± 0.11^{e}	17.75 ± 0.96^{d}	1:2.07
Acarbose	1.72 ± 0.65^{a}	190.05 ± 19.95^{e}	1:110.49

Data are expressed as mean ± SD of two independent experiments done in triplicate. [a-e]Values with different subscript letters along a column are significantly different from each other (Tukey's-HSD multiple range post hoc test, $p < 0.05$)

The effect of alanine substitution on the *in vitro* inhibitory activity of SQSPA against α-glucosidase and α-amylase is presented in Table 4. Based on the results, it was only the substitution at position 1 (AQSPA) that significantly ($P < 0.05$) increased the inhibitory activities of the parent peptide SQSPA against both α-glucosidase and α-amylase but other substitutions resulted in a significantly ($P < 0.05$) lower inhibitory activities against the α-glucosidase but not α-amylase. This is because the peptide analogs, SASPA and SQAPA, had significantly ($P < 0.05$) lower IC_{50} values against α-amylase compared to the SQSPA. Compared to all the peptides, acarbose had a significantly lower IC_{50} value against α-glucosidase but recorded the highest IC_{50} value towards α-amylase. The peptide, SQSAA had the highest IC_{50} values for the inhibition of both α-glucosidase and α-amylase (Table 4) although the data was statistically similar to that of SASPA. In terms of potency ratio for inhibiting the two enzymes, acarbose had the lowest ratio of α-glucosidase to α-amylase inhibition (1: 110.49) while the alanine substitutions on the SQSPA at all positions increased

the ratio from 1:3 to approximately 1: 2 (AQSPA, SQAPA and SQSAA). The peptide analog, SASPA, had the highest potency ratio of 1:1.15.

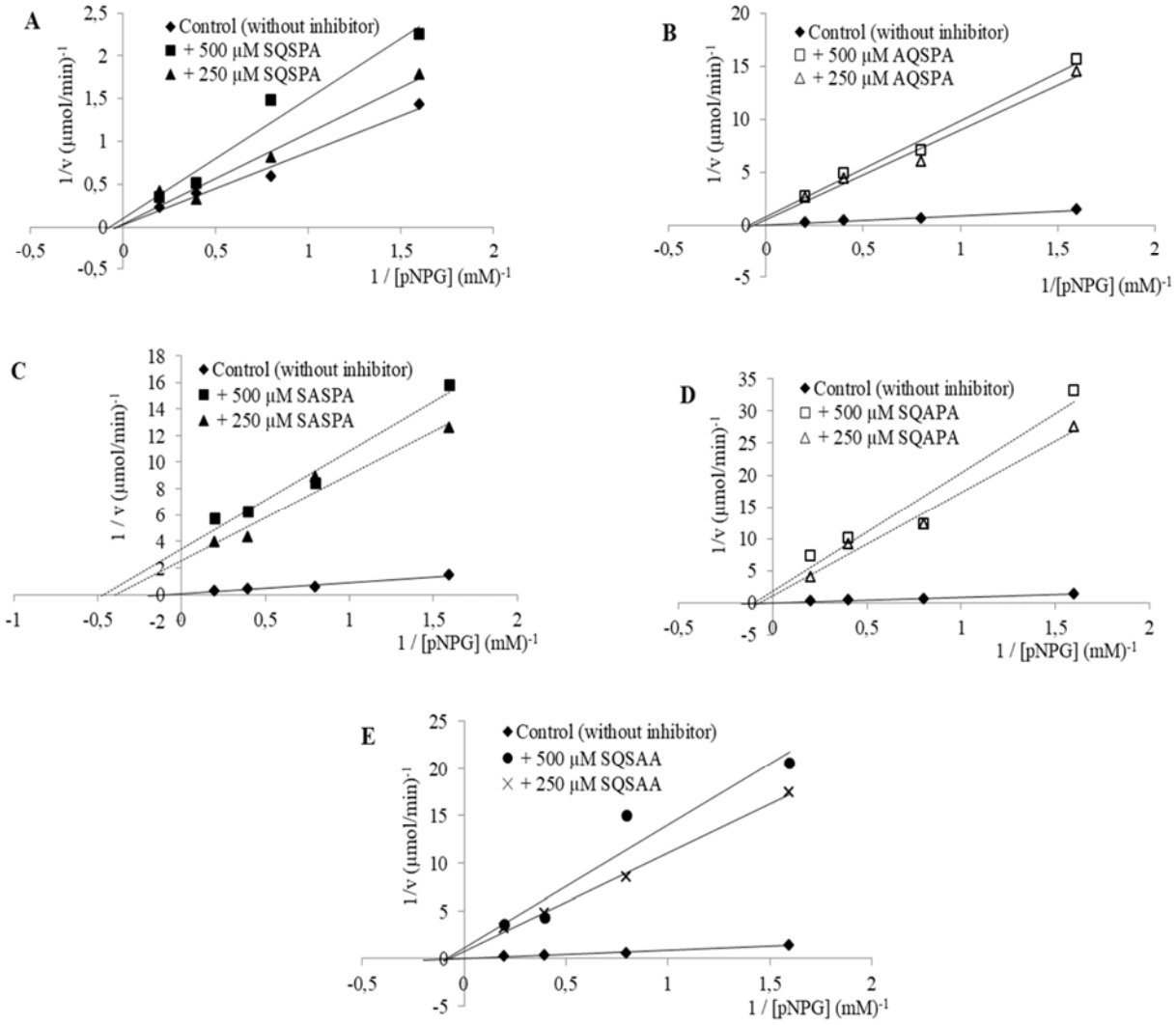

FIGURE 3 Inhibition kinetics of SQSPA (**A**), AQSPA (**B**), SASPA (**C**), SQAPA (**D**) and SQSAA (**E**) against α-glucosidase using Lineweaver-Burke's analysis. Each data point represents a mean of two independent experiments done in triplicates

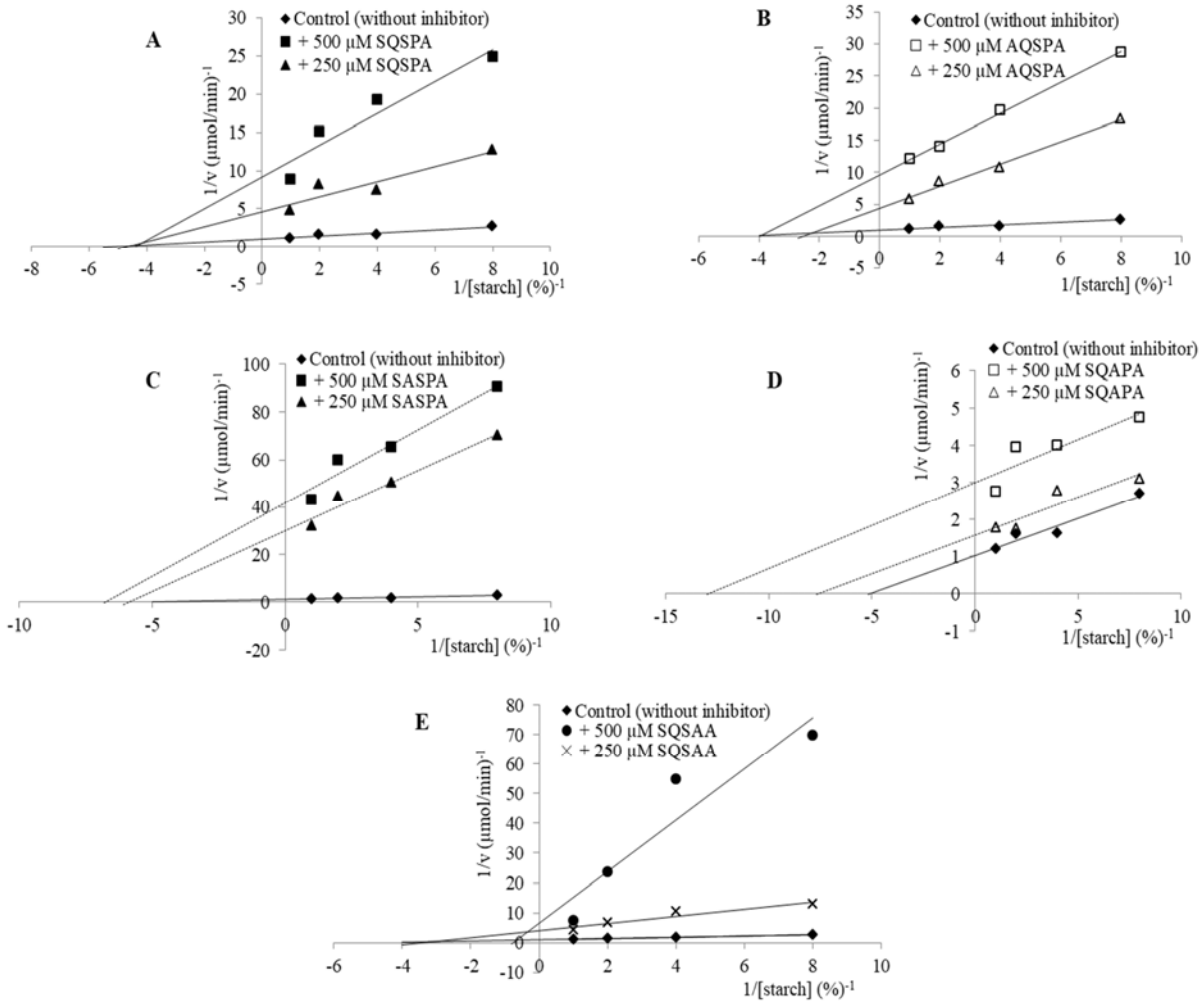

FIGURE 4 Inhibition kinetics of SQSPA (**A**), AQSPA (**B**), SASPA (**C**), SQAPA (**D**) and SQSAA (**E**) against α-amylase using Lineweaver-Burke's analysis. Each data point represents a mean of two independent experiments done in triplicates

Apart from understanding the effects of alanine substitution on the *in vitro* α-glucosidase and α-amylase inhibitory potentials of SQSPA, enzyme inhibition kinetics was also conducted to decipher the role of the substitution on the inhibitory mechanisms of the peptide analogs (Fig. 3 and 4). For α-glucosidase inhibition, the parent peptide SQSPA showed a mixed inhibition pattern but alanine substitution at positions 1, 3 and 4 shifted the inhibition pattern to a pure non-competitive inhibition while the substitution at position 2 changed the inhibition mechanism to an uncompetitive pattern (Fig. 3). In contrast, the alanine substitution at positions 1 and 4 modulated the inhibitory mechanism of SQSPA against α-amylase from non-competitive to mixed inhibition pattern. Moreover, the substitution positions 2 and 3 resulted in an uncompetitive inhibition against α-amylase (Fig.

4). A summary of the inhibitory mechanisms of SQSPA and its analogs against both α-glucosidase and α-amylase is provided in Table 5.

TABLE 5 Summary of the inhibition mechanisms of SQSPA analogs against α-glucosidase and α-amylase

Peptide Ligands	α-Glucosidase	α-Amylase
SQSPA	Mixed	Non-competitive
AQSPA	Non-competitive	Mixed
S**A**SPA	Uncompetitive	Uncompetitive
SQ**A**PA	Non-competitive	Uncompetitive
SQS**A**A	Non-competitive	Mixed

4. DISCUSSION

Even though SQSPA was reported to be a gastrointestinally stable peptide with good α-glucosidase inhibition [10, 21] but, in this study, alanine scanning was used to decipher the functional relevance of each residue and has led to the design of a more potent α-glucosidase inhibitory peptide via substitution at position 1.

For effective α-glucosidase inhibition, a peptide is expected to reach the intestinal lumen in an intact form without being hydrolysed by the digestive enzymes [9]. Therefore, all the SQSPA analogs were subjected to simulated GIT digestion and it was observed that none of the peptide analogs was susceptible to the GIT digestion indicating their suitability as oral therapeutic agents. Subsequently, molecular docking on to α-glucosidase and α-amylase revealed that the binding affinities of the parent peptide SQSPA were enhanced, more prominently; by alanine substitution at positions 1 and 2. It is thus evident that the hydroxyl and amide groups of residues S1 and Q2 respectively, could have hindered the effective interactions between the proteins and the peptide ligands or their close proximity resulted in a conformational change that limits the binding events. In addition to enhancing the binding affinity, the alanine substitution at position 2 also presented the highest number of hydrogen bonds which further supports the functional relevance of this position in the binding of the peptides to the α-glucosidase and α-amylase proteins. The higher binding affinity and lower number of hydrogen bonds observed with substitution at position 1 (AQSPA) compared to substitution at position 2 (SASPA) further supports the fact that hydrogen bond interactions observed in a molecular docking do not always correlates with increased binding affinity and/or biological activities of ligands [10, 11]. In contrast, the substitution at positions 3 and 4 diminished the binding affinity of the SQSPA towards α-glucosidase suggesting that the

residues S3 and P4 were critical to the binding interaction possibly because of the proximity effect and/or the ability of the proline to modulate the flexibility of the peptide and consequently increased the binding affinity towards α-glucosidase. However, unlike some previous studies where α-glucosidase residues K776 [21] and R526 [11] were the critical amino acid residues for the trapping of the inhibitory peptides, our observation with the SQSPA analogs revealed that R283 was the most crucial in this regard. Interestingly however, the foregoing observations from these studies clearly indicate that, irrespective of the binding site, positively charged amino acids could be the main residues for the trapping of the α-glucosidase inhibitory peptides which supports the earlier observations by Zhang et al. [21]. Overall, alanine substitution of SQSPA at the first position led to an analog (AQSPA) that showed better binding affinity and more promising docking parameters toward both α-glucosidase and α-amylase.

Although molecular docking and other virtual screening techniques are presently used as important tools to understand the structure-function relationship of macromolecules, it is always important to validate the *in silico* observations with *in vitro* studies [22]. Interestingly, in the present study, the *in vitro* analysis correlated, to a large extent, with the *in silico* analysis. This is because AQSPA (best binding affinity) with alanine substitution at position 1 demonstrated the most potent *in vitro* α-glucosidase and α-amylase activities than other peptide analogs while the substitution at position 4 (SQSAA had the weakest binding affinity) greatly diminished the activities. This further indicates that S1 in the parent peptide might have hindered the activities whilst the P4 is relevant for enhanced activity. Interestingly, the observed effect of the S1 on the α-glucosidase and α-amylase inhibitory activities of the parent peptide contradicts our previous reports [10] along with others where the presence of a hydroxyl group has been repeatedly demonstrated to be a critical feature of potent α-glucosidase inhibitors [22 – 25]. Perhaps, the nature of the neighbouring chemical environment such as an amide group of glutamine might have an influence in the ability of the hydroxyl group to make effective contribution towards the α-glucosidase inhibition. This could be supported by the lowered α-glucosidase inhibition activity of SQAPA compared to the AQSPA. Conversely, the crucial role of the P4 and to a lesser extent Q2, towards the α-glucosidase inhibitory activity of the peptide further confirms our earlier observation [10] where peptides such as YINQMPQKSREA, KLPGF, TPSPR and PFP [26 – 28] with proline residues showed potent α-glucosidase inhibitions. In fact, the presence of proline within the

peptide sequences has been shown to be relevant for biological activities such as DPP-IV and angiotensin converting enzyme inhibitory effects [29, 30] and it has been linked to the ability of the proline residue to alter the flexibility of the peptides and consequently increase their binding affinity. On the other hand, the α-glucosidase inhibitory activity for the parent peptide SQSPA (IC_{50} = 3.09 ± 0.29 mM) recorded in this study was higher than the previous report of Zhang et al. [22] where the IC_{50} value was reported to be 20 μM suggesting that inter-laboratory variations greatly affect the outcome of the measurement of α-glucosidase inhibitory potency of peptides. With regard to the potency ratio for inhibiting α-glucosidase to α-amylase, no single amino acid residue within the SQSPA sequence could clearly be identified as very influential in altering the ratio. This is because very slight changes were observed with the ratio especially when the data was compared to acarbose. Hence, more studies would be required in this regard especially due to the need to develop an inhibitor with mild-to-moderate α-amylase inhibition and strong α-glucosidase inhibition.

Enzyme inhibition kinetics have been the subject of research efforts to elucidate the mechanism of action of bioactive peptide where the enzyme activity is measured in the presence of different inhibitor concentration with variable substrate concentrations [31]. In the present study, 250 and 500 μM of the peptides were selected because these concentrations are less than $1/10^{th}$ of the K_M value for the enzyme (25 000 μM). Apart from the use of IC_{50} values, inhibitor concentration close the total enzyme concentration or two fold serial dilutions of inhibitor concentration less than $1/10^{th}$ of the K_M value are also valid approaches for deciding the inhibitor concentration for enzyme kinetic studies [32, 33]. Our findings revealed that the alanine substitution greatly alters the mechanism of glucosidase inhibition of the parent peptide, SQSPA (mixed inhibition) where substitutions at positions 1, 3 and 4 led to binding of the peptide at another site, rather than the active site. This suggested conformational change at the active site resulting in lowered α-glucosidase activity whilst substitution at position 2 led to the binding of the peptide to the α-glucosidase-pNPG complex [34]. Moreover, the observations could clearly indicate that the amide side chain of glutamine is responsible for the observed mixed inhibition of the parent peptide, otherwise, the binding mechanism would have been pure non-competitive. A number of bioactive peptides have shown varied mechanism of action such as mixed, competitive, non-competitive, uncompetitive inhibitions toward their enzyme targets [30, 31]. This was also demonstrated in the case of α-amylase inhibition where the amide side chain of glutamine (Q2) and the hydroxyl group of serine (S3) appeared to be responsible for a mixed inhibition

pattern whereas the hydroxyl group of serine (S1) and/or pyrrolidine side chain of P4 might be relevant for the non-competitive inhibition pattern of the SQSPA. Generally, the observed mechanisms of α-glucosidase and α-amylase inhibitory action of the SQSPA and its analogs agreed with the docking experiments where none of the peptides bind at the active site of the enzyme.

The structure-activity analysis of SQSPA suggests that the pyrrolidine side chain of P4 and to a lesser extent, the amide side chain of Q2, are important requirements for the α-glucosidase and α-amylase inhibitory activities of the peptide. Moreover, all amino acid side chains within the peptide sequence could influence the mechanism of α-glucosidase and α-amylase actions with the amide side chain of glutamine (Q2) responsible for a mixed inhibition pattern towards α-glucosidase and along with the hydroxyl group of serine (S3), account for the similar inhibition mechanism against α-amylase. Importantly, the alanine scanning has led to the design of a novel and more potent α-glucosidase inhibitory peptide (AQSPA) and according to the drug discovery pipeline, this peptide would be subjected to biological and cell-culture based assays in our subsequent studies.

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CONFLICT OF INTEREST

There is no conflict of interest in the study

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