The tryptic peptides of hemoglobin for diagnosis of type 2 diabetes mellitus using label-free and standard-free LC-ESI-DMRM

Jie Hong, Shuchen Xin, Rui Min, Yongqian Zhang*, Yulin Deng

School of Life Science, Beijing Institute of Technology, Beijing, 100081, China

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ABSTRACT

N-1-(deoxyfructosyl) valine of β-hemoglobin, commonly referred to as HbA1c, is the “gold standard” for clinical detection of diabetes. Instead of quantifying the full-length HbA1c glycated protein, in the present study, we proposed the peptide-based strategy to quantify the depletion of the tryptic peptides of hemoglobin for the diagnosis of type 2 diabetes mellitus (T2DM). The peptides were discovered and validated as T2DM biomarkers by label-free LC-ESI-DMRM method without reference material. The glucose could react with hemoglobin’s free amino group of N-terminus and ε-amino group of lysine residues and leave the modification on the hemoglobin tryptic peptides. Thus, there are two types of peptides in the hemoglobin: sensitive peptides and insensitive peptides to glucose due to the differential sensitivity of lysine residues to glycation. To discover two types of peptides of hemoglobin, we first developed the assay of liquid chromatography-electrospray ionization mass spectrometry coupled with dynamic multiple reaction monitoring. The protein coverage reaches 94.2%. Moreover, the hemoglobin was incubated with the 500 mmol/L glucose for 20 days, 40 days and 60 days in vitro to screen the sensitive peptides and insensitive peptides to glucose. A total of 14 sensitive peptides and 4 insensitive peptides were discovered. Furthermore, the LC-ESI-DMRM method was also utilized to validate the glucose-sensitive peptides by 40 clinical samples with healthy control individuals (n = 20) and type 2 diabetes mellitus patients (n = 20). Three putative sensitive peptides (LLGVVLYLVLHVFQK, LKVAYLNSNLHGY, LRVDPV/QKK) from the hemoglobin showed excellent sensitivity and specificity based on receiver operating characteristic analysis and were verified as the promising biomarkers for the diagnosis of diabetes mellitus. And one peptide (LLVYVPPWQR) was found as glucose-insensitive peptide. Taken together, the findings of this study suggest that quantification of hemoglobin tryptic peptides using label-free and standard-free LC-ESI-DMRM is an alternative method for the diagnosis of T2DM, which could be combined with other MS-based blood biomarkers for diagnosis of multiple diseases in MS single shot.

1. Introduction

Diabetes is a group of endocrine and metabolic diseases characterized by chronic hyperglycemia [1]. If blood glucose is not properly controlled, it would cause a variety of complications, such as renal failure [2], cardiovascular disease [3] and nervous system disease [4], etc. Diabetes mellitus can be diagnosed clinically by fasting plasma glucose [5], oral glucose tolerance test [6] and glycated serum albumin [7,8], etc. Glycated serum albumin (GA) level can reflect the average glucose level of patients in the past 2–3 weeks, which is not affected by the fluctuation of temporary blood glucose concentration [9,10]. In 2015, the American Diabetes Association (ADA) issued a standard for diabetic diagnosis and treatment that clearly defined N-1-(deoxyfructosyl) valine of β-hemoglobin (HbA1c) as the “gold standard” for evaluating blood glucose control. HbA1c can reflect the blood glucose level of diabetic patients within 2–3 months and be used to evaluate long-term blood glucose control in diabetic patients [11].

Glycation is a naturally occurring and non-enzymatic catalyzed reaction in vivo or in vitro, which is also called Maillard reaction [12]. In the blood, the carbonyl group of reducing sugar (such as glucose or fructose or mannose) can react with ε-amino group of the lysine residues and the ε-amino group at the N-terminus of a protein and form a reversibly chemical adduct referred to as the Schiff base. The thermally unstable Schiff base undergoes rearrangement reaction to produce the
Fig. 1. The workflow diagram that shows the discovery and validation of hemoglobin tryptic peptides as putative biomarkers for T2DM diagnosis. (A) The discovery of glucose-sensitive peptides as potential biomarker candidates and glucose-insensitive peptides by in vitro study. (B) The validation of putative biomarkers for T2DM diagnosis by clinical samples.

Amadori product [13,14], 1-amino-1-deoxy-2-ketose, which is an early glycation product [15]. Then, the Amadori product undergoes a series of reactions such as condensation, dehydrogenation, rearrangement and isomerization of active intermediates, resulting in irreversible advanced glycation end products (AGEs) [16,17]. AGEs play an important role in the occurrence and development of diabetes and its complications. The concentration of AGEs significantly increased with the elevation of blood glucose in diabetic patients, revealing the correlation between AGEs and diabetes [18]. It was reported that the concentrations of two amide-AGEs in apolipoprotein A-II and human serum albumin were significantly higher in T2DM [19]. Therefore, AGEs are possible to become new biomarkers for the diagnosis of diabetes. Instead of studying the AGEs, glycosylated or glycated peptides were also investigated by mass spectrometry and correlated with the progress with T2DM. For example, VHLTPE and 1-deoxyfructosyl-VHLTPE, which are N-terminal peptides of the β chains of hemoglobin for quantification of HbA1c, were considered as the marker of T2DM [20]. Moreover, the roles of deoxyfructosyl, carboxymethyl, and carboxyethyl based hemoglobin modifications have been studied in clinical prognosis and diagnosis of type 2 diabetes mellitus [21]. In another study, glycation sites of serum albumin, serotransferrin, and haptoglobin appear to be promising biomarkers for the early diagnosis of T2DM [22]. Twenty Amadori peptides are quantified and validated as new biomarker candidates for T2DM based on boronic acid affinity chromatography coupled with LC-ESI-MS [23]. Despite these accomplishments, challenges in the diagnosis of T2DM remain by mass spectrometry due to the AGEs’ low concentration and diverse structures. Quantifying the AGEs or glycated peptides often requires enrichment techniques or purification by affinity chromatography, which affects throughput and reproducibility. In some cases, the reference material or even the isotope-labeled standard was needed accordingly by mass spectrometry. Thus, it is urgent to develop a label-free and standard-free method for the diagnosis of T2DM without time-consuming sample pretreatment.

In our previous study, we put forward a strategy for diagnosis of diabetes based on the fact that reducing sugars or aldehydes may react non-enzymatically with amino groups of proteins through Maillard reaction [24]. Three peptides from human albumin, the highest abundance protein in the plasma, were sensitive to glucose and could be regarded as putative biomarkers for type 2 diabetes mellitus (T2DM). It was reported that the albumin only has a half-life of approximately 15–20 days [25,26]. The half-life of hemoglobin is three-fold than that of albumin (the half-life of hemoglobin is approximately 50–60 days) [27]. In the present work, we propose that the longer half-life protein has much more time to react with reducing sugars or aldehydes in the blood. The level of glucose-sensitive peptides digested from the hemoglobin decreases more in the T2DM group than healthy group. We developed the label-free and standard-free LC-ESI-DMRM method to determine the depletion of unmodified hemoglobin tryptic peptides instead of the glycated protein or the post-translational modification (PTM) peptides as the biomarkers of diabetes. The workflow chart illustrating the study design is shown in Fig. 1. The glucose could react with hemoglobin’s free amino group of N-terminus and ε-amino group of lysine residues and leave the modification on the hemoglobin tryptic peptides in vitro. Thus, there are two types of peptides in the hemoglobin: glucose-sensitive peptides and glucose-insensitive peptides due to the differential sensitivity of lysine residues to glycation. A total of fourteen sensitive peptides and four insensitive peptides were discovered by in vitro study. Through the screening by clinical samples, we found that three glucose-sensitive peptides and one glucose-insensitive peptide from hemoglobin are expected to become the putative biomarkers for the diagnosis of diabetes mellitus by mass spectrometry. The mass spectrometry-based peptide assay is not only a label-free and standard-free method, but also suitable for clinical testing due to its ease of use. Moreover, it offers excellent sensitivity and accuracy for the diagnosis of T2DM. Furthermore, these peptides could also be combined with other MS-based blood biomarkers for diagnosis of multiple diseases in MS single shot, which play critical roles in prediction, diagnosis and treatment in precision medicine.

2. Material and Methods

2.1. Chemicals and reagents

Urea, dithiothreitol (DTT) and ammonium carbonate (NH₄HCO₃) were purchased from Sigma-Aldrich (Steinheim, Germany). Iodoacetamide (IAA) was purchased from Biodee biotechnology Co. Ltd (Beijing, China). Sequencing-grade modified trypsin was purchased from Promega (Madison, WI, USA). HPLC-grade formic acid and acetonitrile (ACN) were purchased from Fisher Scientific (Edmonton, Canada). Water was obtained from a Millipore Milli-Q Plus purification system (Bedford, MA, USA).

2.2. Hemoglobin glycation in vitro

Glycation of hemoglobin was incubated with glucose according to the previous protocol with slight modification [24]. For glycation of hemoglobin, 100 mM hemoglobin was incubated with 500 mM glucose in 10 mM PBS (pH 7.2) at 37 °C for 20 days, 40 days and 60 days in vitro,
which screened the sensitive peptides and insensitive peptides to glucose. Three parallel samples were prepared at each incubation time in the hemoglobin glycation group, and the hemoglobin without the addition of \( \beta \)-glucose under the same conditions was used as a blank control. All solutions were added sodium azide as a preservative.

2.3. Clinical samples preparation

The whole blood sample (\( <3 \) mL) was collected in vacutainer tubes with K2EDTA as an anticoagulant from the Beijing Institute of Technology Hospital, Beijing, China. The forty samples were grouped into healthy controls (\( n = 20 \)) and T2DM groups (\( n = 20 \)) based on the value of HbA1c. The American Diabetes Association (ADA) and the World Health Organization (WHO) recommend T2DM is diagnosed at an HbA1c value more than or equal to 6.5%. The clinical sample information was listed in Table S1. This study was performed in accordance with the principles of the medical ethics of the Beijing Institute of Technology. Approximately 1 µl whole blood was denatured in 8 M urea in the water bath at 37 °C for 1 h, alkylated with 50 mM iodoacetamide for 45 min at room temperature, and then diluted in a solution of 50 µM ammonium bicarbonate to decrease urea concentration to 1 M. Trypsin (enzyme/proteins ratio = 1:50 w/w) was added and the sample was incubated at 37 °C in the water bath for 18 h. Trypsin remaining in the sample was deactivated by the addition of 1% formic acid.

2.4. Identification of hemoglobin by LC-ESI-QTOF

Agilent 1290 series HPLC system was directly coupled to an Agilent 6460 Series Triple quad MS (QQQ). The chromatography system and parameters were fully consistent with that of LC-ESI-QTOF. The tryptic digests from approximately 1 µg hemoglobin were quantified by LC-ESI-QQQ. The QQQ ionization mode was positive electrospray and DMRM scan type was selected. The transitions of digested hemoglobin tryptic peptides were shown in Table S2. The nebulizer pressure was 35 psi. Drying gas flow and temperature was 7 L/min and 300 °C, respectively. Sheath gas flow and temperature was 11 L/min and 250 °C, respectively. The capillary and Delta EMV are 3500 V and 400 V, respectively. LC-ESI-DMRM was used to screen the sensitive and insensitive peptides digested from hemoglobin. For each peptide, at least two or three transitions were selected for integration and the highest peak area was used for quantification. For in vitro study, the ratios of peak areas of the tryptic peptides of hemoglobin in the incubation group to the control group were calculated. The ratios close to 1 were regarded as glucose-insensitive peptides and the ratios less than 1 were regarded as glucose-sensitive peptides. For in vivo study, the ratios of peak areas of glucose-sensitive peptides to glucose-insensitive peptide LLVVYPWTQR were calculated. Total ion chromatographs of hemoglobin alpha subunit (Fig. 2A) and beta subunit (Fig. 2B) were acquired by LC-ESI-DMRM. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.5. Quantification of hemoglobin tryptic peptides by LC-ESI-DMRM in vitro and in vivo

For quantification of hemoglobin tryptic peptides, Agilent 1290 series HPLC system was directly coupled to an Agilent 6460 Series Triple quad MS (QQQ). The chromatography system and parameters were fully consistent with that of LC-ESI-QTOF. The tryptic digests from approximately 1 µg hemoglobin were quantified by LC-ESI-QQQ. The QQQ ionization mode was positive electrospray and DMRM scan type was selected. The transitions of digested hemoglobin tryptic peptides were shown in Table S2. The nebulizer pressure was 35 psi. Drying gas flow and temperature was 7 L/min and 300 °C, respectively. Sheath gas flow and temperature was 11 L/min and 250 °C, respectively. The capillary and Delta EMV are 3500 V and 400 V, respectively. LC-ESI-DMRM was used to screen the sensitive and insensitive peptides digested from hemoglobin. For each peptide, at least two or three transitions were selected for integration and the highest peak area was used for quantification. For in vitro study, the ratios of peak areas of the tryptic peptides of hemoglobin in the incubation group to the control group were calculated. The ratios close to 1 were regarded as glucose-insensitive peptides and the ratios less than 1 were regarded as glucose-sensitive peptides. For in vivo study, the ratios of peak areas of glucose-sensitive peptides to glucose-insensitive peptide LLVVYPWTQR were calculated from 40 clinical samples. The ratios among the clinical samples were imported into Perseus (v.1.6.2.3) software and then log-transformed with base 2 for principal component analysis (PCA) and cluster analysis. PCA was performed and visualized using default settings. The number of components is 5 and the method is Benjamini-Hochberg using cutoffs set an FDR of 0.05. The hierarchical clustering analysis was done using Pearson correlation distance and average linkage, preprocessing with k-means for 300 clusters. The hemoglobin protein three-dimensional structures utilized in this study were obtained from the PDB (accession codes:6BBS). The 3D graph was generated from PyMol Molecular Graphics System.

2.6. Quantification and statistical analysis

For each clinical sample, the peak areas of glucose-sensitive peptides from hemoglobin were normalized against the peak area of glucose-
insensitive peptide LLVVYPWTQR to obtain the relative quantification results, which were expressed as the mean ± standard deviation. For each peptide, statistical evaluations were performed using Student’s t-test between the healthy controls and T2DM patients. P values of less than 0.05 were considered significant statistically. The receiver operating characteristic (ROC) curves were generated for putative biomarkers in clinical samples using GraphPad Prism 8. The mass spectrometry data were deposited to the iProx database (accession number: IPX 0002741000).

3. Results and discussion

3.1. Identification of hemoglobin tryptic peptides by LC-ESI-QTOF and LC-ESI-QQQ

The tryptic peptides of hemoglobin were identified by LC-ESI-QTOF firstly. As shown in Fig. 2A and Fig. 2C, the hemoglobin protein coverage reaches 94.2%, although the peptides TNVK, GHGKK, YR in the hemoglobin alpha subunit and the peptides VK and AHGKK in beta subunits in red are not detected. Of note, the four peptides TYFPHFDLSHGSAQVK, VADALTNAHVDDMPNALSALSALDHAK, LLSHCLLTVLAHLPAEFTPAVHASLDK and GTFATLSELHCDK in brown have relatively poor MS response probably due to the large molecular weight or low ionization efficiency. It was noteworthy, these four peptides can still be identified in clinical samples by LC-ESI-DMRM since the novel assay of liquid chromatography electrospray ionization mass spectrometry coupled with dynamic multiple reaction monitoring (LC-ESI-DMRM) is developed to quantify the tryptic peptides of hemoglobin.

3.2. Screening of glucose-insensitive peptides

For glycation of hemoglobin, 100 mM hemoglobin was incubated with 500 mM glucose in 10 mM PBS (pH 7.2) at 37 °C for 20 days, 40 days and 60 days in vitro to screen the glucose-insensitive peptides (Fig. 3A) and glucose-sensitive peptides (Fig. 3B). (A) The ratios of peak areas of hemoglobin peptides VNVDEVGGEALGR, LLVVYPWTQR, SAVTALWGK, and TYFPHFDLSHGSAQVK in the experimental and control groups at different incubation days almost have no change. (B) The ratios of peak areas of four glucose-sensitive peptides at different incubation days were down-regulated. The ratios of peak areas of LRVDPVNFK and VVAGVANALAHKYH began to reduce significantly at day 40, while the ratios of peak areas of four glucose-insensitive peptides LRGDNVVCVLAHHFGK was decreased at day 60.

For glycation of hemoglobin, hemoglobin was incubated with glucose for 20 days, 40 days and 60 days in vitro. After the digested samples were performed in the LC-ESI-DMRM, the peak area ratios of the tryptic peptides in the glycated hemoglobin group to the control group were calculated. The ratios close to 1 were regarded as glucose-insensitive peptides. As shown in Fig. 3A, the four peptides LLVVYPWTQR, VNVDEVGGEALGR, SAVTALWGK, and TYFPHFDLSHGSAQVK were selected as glucose-insensitive peptides from the 22 tryptic hemoglobin tryptic peptides in vitro. The ratios of four peptides almost didn’t change and were independent of the incubation days in the glycated hemoglobin. Furthermore, the four peptides had good response in QQQ and were able to be stably detected and quantified in 24 consecutive injections. The coefficient of variation (CV) of peak areas of four glucose-insensitive peptides was 6.39%, 7.09%, 9.83% and 9.91%, respectively. After tested by clinical samples, the CV of peak areas of peptide LLVVYPWTQR in healthy control and T2DM groups was 9.82%. The peptide LLVVYPWTQR was verified as glucose-insensitive peptide in vivo. Furthermore, another reason why chose LLVVYPWTQR as glucose-insensitive peptide was that no post-translational modification was found for peptide LLVVYPWTQR through the in vitro and in vivo experiments [26]. As shown in Fig. 6, the peptide LLVVYPWTQR was located at the center of the subunits of...
hemoglobin in the three-dimension structure, which might contribute to the insensitivity to the glucose. Considering that the hemoglobin concentration was slightly different in the cohorts [28], the peptide LLVVYPWTQR cleaved from the hemoglobin itself could be a promising candidate to normalize the levels of glucose-sensitive peptides from the hemoglobin in healthy individuals and T2DM patients.

3.3. Screening of sensitive peptides as putative biomarker candidates

The glucose can react non-enzymatically with N-terminal α-amino groups of proteins or ϵ-amino groups of lysine residues to initiate the advanced glycation through Maillard reaction. Thus, if the ratios of peak areas of peptides between the experimental group and the control group were decreased as incubation days went by, these peptides were regarded as glucose-sensitive peptides. As listed in Table S4, the 14 peptides were vulnerable to be modified and regarded as glucose-sensitive peptides. As shown in Fig. 3b, the peak areas of some of peptides began to reduce significantly at day 40, while others were decreased at day 60, which might be associated with the lysine’s position and surrounding in the hemoglobin spatial structure. It was speculated the glucose is easier to attack the lysine residues of the glucose-sensitive peptides located on the outer surface of the protein.

3.4. Quantification and validation of putative biomarkers for diabetes mellitus

To verify the 14 glucose-sensitive peptides and 4 glucose-insensitive peptides, a total of 40 clinical samples were used to screen the putative biomarkers. The peak areas of glucose-sensitive peptides were normalized against the peak area of glucose-insensitive peptide (LLVVYPWTQR) to obtain the relative quantification results. The horizontal line in the middle of each box indicates the median, and the top and bottom borders of the box mark the 75th and 25th percentiles, respectively. The whiskers above and below the boxes represented the 90th and 10th percentiles. p-value was determined by an unpaired Student’s t-test. **p < 0.01, ***p < 0.001 vs control group.

Fig. 5. The box plot and ROC analysis of the five putative peptide biomarkers for T2DM with the glucose-insensitive peptide (LLVVYPWTQR). The peak areas of glucose-sensitive peptides from hemoglobin were normalized against the peak area of glucose-insensitive peptide (LLVVYPWTQR) to obtain the relative quantification results. The horizontal line in the middle of each box indicates the median, and the top and bottom borders of the box mark the 75th and 25th percentiles, respectively. The whiskers above and below the boxes represented the 90th and 10th percentiles. p-value was determined by an unpaired Student’s t-test. **p < 0.01, ***p < 0.001 vs control group.

The ratios of peak areas of five glucose-sensitive peptides to LLVVYPWTQR were shown in Fig. 5 between the healthy control and T2DM groups when the LC-ESI-DMRM assay was applied to the clinical samples. The concentrations of five peptides of the 14 putative biomarker candidates were significantly decreased in the T2DM group compared with the healthy control group, which indicated that five glucose-sensitive peptides might be served as potential biomarkers for the diagnosis of T2DM. Rather than quantify the depletion of the unmodified peptides as biomarkers, there are numerous studies to investigate the hemoglobin peptides with various post-translational modifications for T2DM. The challenge of studying the modified peptides is their diverse chemical structures and relatively low concentration in the blood. For example, three kinds of glycated hemoglobin peptides were determined by pseudotargeted parallel reaction...
monitoring (PRM) method [21]. Although the T2DM patients were fully distinguished from healthy controls, it was still required to build the pseudotargeted PRM library for modified hemoglobin using time-consuming multiple enzyme digestion. In addition, the human hemolysate was incubated with isotopically labeled-glucose to comprehensively profile the glycated peptides and discover glycation sites, facilitating the comparison of glycation with different glycomic states [29]. However, the boronate affinity chromatography was a necessary step to enrich the low-abundance glycated peptides prior to LC-MS analysis. Therefore, it was worth noting that our unmodified peptide-based assay is not only very simple and convenient during the sample preparation, but also is a label-free and standard-free method suitable for clinical analysis.

### 3.5. Diagnostic power based on ROC curve

Receiver operating characteristic (ROC) analysis is a valuable tool for evaluating the diagnostic power [30]. The ROC curve is illustrated based on sensitivity and specificity scores. Through the quantification of glucose-sensitive peptides on clinical samples, we explored their potential as biomarkers to classify the healthy group and T2DM group. Generally, the higher the AUC score, the better the peptides perform for the diagnosis of T2DM. As shown in Fig. 6 and Table 1, three peptides’ scores of AUC were greater than 0.9, which meant the biomarkers had high accuracy. Furthermore, the top three peptides were correlated to the values of HbA1c of clinical samples in Fig. S1. In 2015, the American Diabetes Association (ADA) issued a standard for diabetic diagnosis. The normal HbA1c value for (non-diabetic) adults is below 6.0. The value of HbA1c is 6.5% or above for type 2 diabetes. With the increase of HbA1c score is greater than 0.9 between the healthy group and T2DM group. In addition, one peptide (LLVVYPWTQR) was validated as glucose-insensitive peptide by clinical samples. Taken together, the findings suggest that these peptides could be promising biomarkers for the diagnosis of T2DM.

### Table 1

Parameters of ROC analysis of five putative peptide biomarkers between healthy control (n = 18) and T2DM group (n = 19).

| Putative biomarkers   | Cut-off point | Sensitivity (%) | Specificity (%) | AUC  |
|-----------------------|--------------|-----------------|-----------------|------|
| VVAGVANALAHKYH        | 0.014        | 100.00          | 100.00          | 1.00 |
| LRVDPVNFK             | 0.069        | 89.47           | 88.89           | 0.91 |
| LLGNLVCVLAHHFGK       | 0.046        | 84.21           | 94.44           | 0.91 |
| KVLGAFSGDLHHJDLNK     | 0.017        | 68.42           | 94.44           | 0.84 |
| LSHELVTLAALPHAPFPVHASLDK | 0.004  | 63.16           | 83.33           | 0.78 |

* The ratios of peak areas of putative peptide biomarkers to LLVVYPWTQR in each clinical case.

* Area under curve (AUC): 0.5–0.7 means the peptide has low accuracy; 0.7–0.9 means the peptide has medium accuracy; > 0.9 means the peptide has high accuracy.

in our case, overnight digestion is a tedious step during the sample preparation. Thus, the rapid protein digestion technique is required. The fast microwave-assisted digestion [32] and on-line protein digestion [33] are the possible solutions. Furthermore, fast and accurate quantification of the peptides’ level is required by liquid chromatography coupled with mass spectrometry. Although the evolution of ultra-high-pressure liquid chromatography has led to ~10-fold increases in speed of analysis [34], it is still required to develop the high-throughput assay for peptide-based biomarkers in routine clinical analysis. Another emerging technology using direct infusion by data-independent acquisition mass spectrometry has also attracted our attention, which can achieve fast quantification without liquid chromatography [35]. As liquid chromatography and mass spectrometry coevolve, the automated high-throughput clinical analysis of peptide-based biomarkers could be realized in the near future.

### 4. Conclusions

The present study offers a label-free and standard-free LC-ESI-DMRM method to discover and validate the putative peptide biomarkers for the diagnosis of T2DM. The hemoglobin was chosen due to its high abundance and long half-life in the blood. Through the hemoglobin glycation experiment in vitro, 14 glucose-sensitive peptides and 4 glucose-insensitive peptides were found from the glycated hemoglobin. Then, these peptides were verified by a total of 40 clinical samples. Three putative peptides (LRVDPVNFK, LLGNLVCVLAHHFGK and VVAGVANALAHKYH) were quantified with a significant difference and AUC score is greater than 0.9 between the healthy group and T2DM group. In addition, one peptide (LLVVYPWTQR) was validated as glucose-insensitive peptide by clinical samples. Taken together, the findings suggest that these peptides could be promising biomarkers for the diagnosis of T2DM.

### Authors’ contributions

Jie Hong: Data curation, Formal analysis, Investigation. Shuchen Xin: Methodology, Investigation. Min Rui: Visualization, Formal analysis. Yongqian Zhang: Conceptualization, Data curation, Project administration, Writing-original draft & editing. Yulin Deng: Conceptualization, Supervision.

### Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2021.101985.

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