Establishment of Hypoglycemic Agent Screening Method Based on Human Glucokinase[1]

CHOU-FEI WU, YANG XU[2], YONG TAO, AND JI-YAN YANG

Key Laboratory of State Food Science and Technology, Jiangxi-OAI Joint Research Institute, Nanchang University, Nanchang 330047, Jiangxi, China

Objective To establish a reliable platform for screening glucokinase activators (GKAs) *in vitro*. **Methods** Pancreatic glucokinase (PGK) protein expressed in a prokaryotic expression system as a histidine-tagged fusion protein from *Homo sapiens* was produced. Then, response surface methodology (RSM) was used to optimize the microplate-based GKA screening platform. In the first step of optimization with Plackett-Burman design (PBD), initial pH, reaction time and $MgCl_2$ were found to be important factors affecting the activity ratio of GKA (RO-28-1675) significantly. In the second step, a 2^3 full factorial central composite design (CCD) and RSM were applied to the optimal condition determination of each significant variable. A second-order polynomial was determined by a multiple regression analysis of the experimental data. **Results** The following optimal values for the critical factors were obtained: initial pH 0 (7.0), reaction time-0.63 (13.7 min) and $MgCl_2$ 0.11 (2.11 mmol/L) with a predicted value of the maximum activity ratio of 34.1%. **Conclusion** Under the optimal conditions, the practical activity ratio is 34.8%. The determination coefficient (R^2) is 0.9442, ensuring adequate credibility of the model. LLAE3, extracted from *Folium nelumbinis* in our laboratory, has prominently activated effects on PGK.

Key words: Screening mothod; Human pancreatic glucokinase; Protein expression; Glucokinase activators; Response surface methodology

INTRODUCTION

Future therapy for type 2 diabetes focuses on three key aspects: insulin resistance, defective insulin secretion and increased hepatic glucose production. Glucokinase (GK), a member of the hexokinase family, found in the liver, participates in the control of blood glucose homeostasis. In pancreatic beta cells, GK serve as a glucose sensor and regulates insulin release. These particular features make GK a strong potential target for the pharmacological treatment of type 2 diabetes[1-2].

Developments over the past decade, such as establishment of a combined genetic, biochemical and cell-biological approach to the quantification of functional and structural changes of human GK, resulting from MODY-2[3], the determination of GK mutations related to maturity onset of diabetes of the young (MODY)[4], and the discovery of the function of GK regulatory protein[5], have significantly enhanced our understanding of GK structure and function. In the study on the structural, kinetic, and molecular genetic features of GK, more and more small allosteric activators of GK have been discovered[1,6]. For instance, RO-28-1675 increases the *V*max of GK by a factor of about 1.5 and decreases the substrate concentration at 0.5 *V*max ($[S]_{0.5}$) for glucose from 8.6 mmol/L to 2.0 mmol/L at a concentration of 3 μmol/L. Since GKAs are proliferated, it is necessary to construct a reliable high-throughput way for screening GKAs. Such action may help develop a potential functional factor for lowing blood glucose.

RSM is a powerful and efficient mathematical approach widely applied in the optimization of fermentation process[7-10], shedding light on the interaction between variables and providing information necessary for the optimization of design and process and giving multiple responses at the same time.

In the context of such contributions, we developed a rapid microplate-based way for evaluating GKAs and a reliable system for screening GKAs using RSM method for the first time. In order

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[2]Correspondence should be addressed to Yang XU, Tel: 86-791-8329479. Fax: 86-791-8333708. E-mail: xuyang@ucu.edu.cn

Biographical note of the first author: Chou-Fei WU, male, born in 1980, postgraduate student at the school of Life Science, Nanchang University, majoring in microbiology.

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to mimic the pathological and physiological status in diabetic patients *in vivo* and make the screening platform more reliable, we successfully constructed a prokaryotic expression vector 6HisT-pRSET-PGK and obtained a purified human PGK protein with a strong enzymatic activity. In the process of establishing the screening method, RO-28-1675, one commonly available GKA, was used as a positive control. The 96-well plate screening model also makes it possible to rapidly examine a large number of samples in the presence of GKAs in a very small sample volume. Such action may help to pace with the development of potential hypoglycemic agents and therefore treat diabetes mellitus and its related complications.

MATERIALS AND METHODS

Reagents

Total RNA, extracted from healthy human pancreatic tissue, was purchased from Shanghai Genomics (China). ATP, β-NADP, G-6-PDH, and β-D (+) glucose were purchased from Sigma (USA). DNA restriction enzymes, AMV reverse first-strand cDNA synthesis kit, DNA purification kit, DNA gel extraction kit, PrimerSTAR^{TM} HS DNA polymerase, and Taq DNA polymerase were purchased from TaKaRa (China). Ni-NTA His.bind resin was purchased from Merck (USA). RO-28-1675 was a gift from doctor Liu. Other reagents were of analytical purity. Deionized water was used for the preparation of all solutions.

Microorganism, Medium, and Culture Condition

Escherichia coli strain DH5α and *E. coli* BL21 were thoroughly used as host strains for subcloning and gene expression, respectively. They were cultured at 37 ℃ on Luria-Bertani medium [5 g/lL yeast extract, 10 g/L peptone, 10 g/L NaCl].

Construction of Expression Vector

A pair of gene-specific primers based on the *glucokinase* gene sequence NM_000162 was used: forward primer (5′-gggaattccatatgctggacgacaga-gccaggatgg-3′; 36-mer), reverse primer (5′-tacggatcctcactggcccagcatacaggcc-3′; 31-mer). The forward primer has a NdeI restriction site and corresponds to sequence of the end of the initiation codon. The reverse primer has a *Bam*HI restriction site and corresponds to the sequence of the end of the stop codon. RT-PCR was performed according to the instructions of the AMV reverse first-strand cDNA synthesis kit

Plasmid DNA extraction from *E. coli*, DNA restriction, ligation, and *E. coli* transformation were carried out as previously described[11]. Expression plasmid 6HisT-pRSET containing *ampicillin* gene, T7 promoter and 6-his tag, was a gift from doctor Tao[12]. 6HisT-pRSET-PGK expression vector was constructed (Fig. 1).

Expression and Purification of the Fusion Protein

The plasmid 6HisT-pRSET-PGK was transformed into *E. coli* BL21. Recombinant *E. coli* BL21 cells containing the protein expression vector were grown in 250 mL of LB media in the presence of ampicillin (100 mg/mL) at 37 ℃. Production of a target protein was induced by addition of 0.75 mmol/L IPTG when the optical density of the culture broth at 600 nm reached 0.6. Cells were incubated for an additional 3 h and harvested by centrifugation at 4000 rpm for 15 min. The harvested cells were washed twice with 10 mL of phosphate-buffered saline (PBS, pH 7.4). The cells were resuspended in 10 mL of 50 mmol/L Tris-HCl (pH 8.0) and then destroyed by sonication. The crude cell lysate was obtained by centrifugation at 1 3200 rpm for 15 min. The hexahistidine-tagged proteins were purified using Ni-NTA His.bind resin.

Purity, Concentration, and Enzymatic Assay of 6HisT-pRSET-PGK Protein

Recombinant protein was of a 100% purity, as assessed by SDS-PAGE analysis. In order to determine the concentration of PGK protein, Bradford quantification of protein concentration was carried out as previously described[13]. A 3.00 mL reaction mixture containing the final concentrations of 60 mmol/L Tris, 2.0 mmol/L $MgCl_2$, 4.0 mmol/L ATP, 12.0 mmol/L β-D(+) glucose, 0.9 mmol/L β-NADP and 100 U/mL of 5 μL G-6-PDH, was used to assay the enzymatic activity of PGK. According to the increase in $OD_{340\ nm}$ for approximately 10 minutes at 30 ℃, pH 9.0 of both the test and black, we calculated the enzymatic activity with the formulas: U/mL enzyme solution=($OD_{340\ nm}$/min test- $OD_{340\ nm}$/ min black)(3)/ (6.22)(0.1) and U/mg enzyme protein=U/mL enzyme/ (mg protein/mL enzyme).

Plackett-Burman Design (PBD)

In this part, PBD was used to evaluate the relative importance of each variable of the screening system. This design did not consider the interaction effects among the variables and was used to screen the important variables affecting the activity ratio of RO-28-1675, which was used as a standard control. The experimental design for the screening system is shown in Table 1. Each variable was set at two levels:

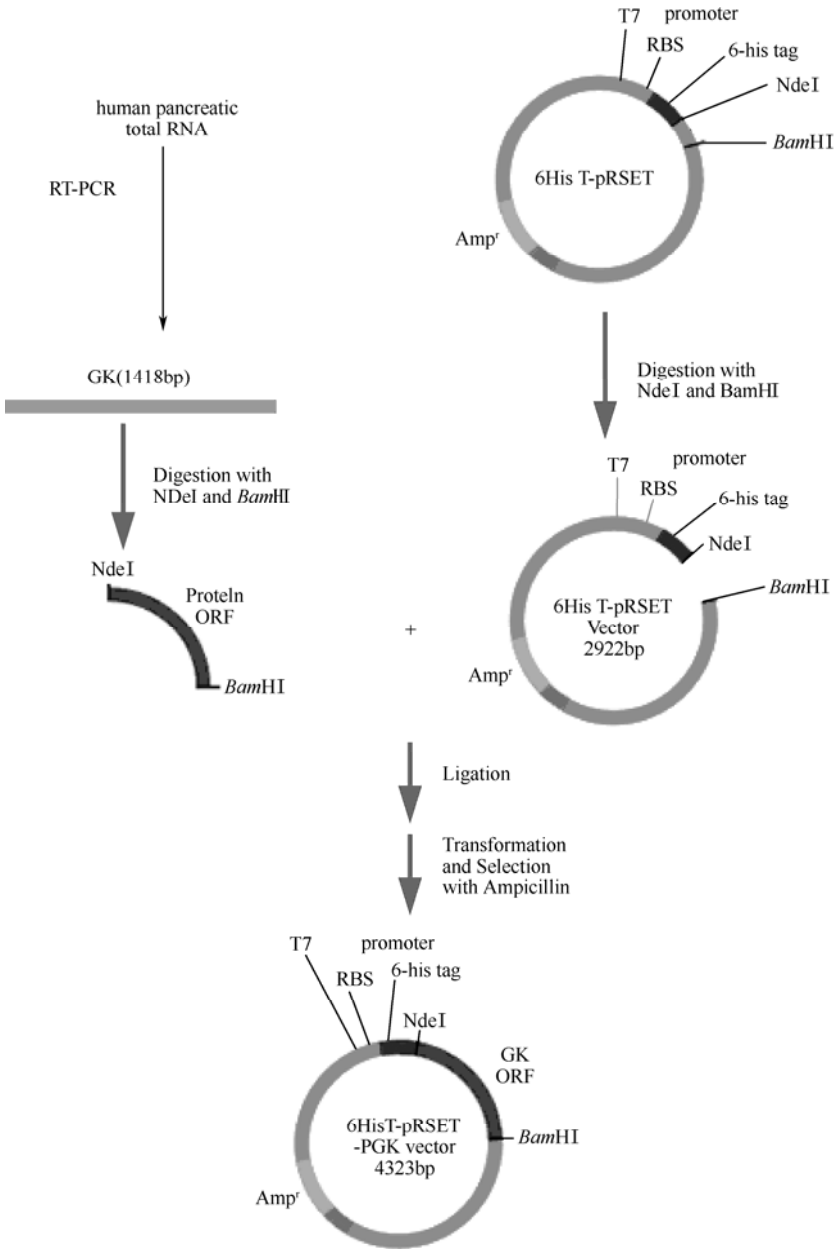

FIG. 1. Construction of 6HisT-pRSET-PGK expression vector. The fragment PGK of PCR product was digested with NdeI + *Bam*HI, ligated to 6HisT-pRSET, digested with NdeI + *Bam*HI again to generate 6HisT-pRSET-PGK.

high level and low level. The high level of each variable was set far enough from the low level to identify which conditions of the system have significant influence on the activity ratio of RO-28-1675. The design matrix (Table 1) was developed using a Design-Expert 6.0.5.

Central Composite Design (CCD)

To obtain the optimum levels of the reaction conditions, a CCD was adopted to optimize the major variables (initial pH, reaction time and $MgCl_2$), which were selected through PBD. Coded levels for independent variables are presented in Table 2.

A 2^3-factorial CCD, with 6 axial points (a=1.682) and 6 replications at the central points (n_0=6) leading to a total number of 20 experiments, was employed (Table 2) for the optimization of the three chosen variables. Activity ratio (Y, %) was used as the dependent output variable. The second degree polynomials, Eq.(1), including all interaction terms, were

TABLE 1

Experimental Design Using PBD for Screening of Reaction Conditions

Run	Variables								Activity Ratio (%)
	A	B	C	D	E	F	G	H	
1	+1	- 1	+1	- 1	- 1	- 1	+1	+1	25.1
2	+1	+1	- 1	+1	- 1	- 1	- 1	+1	28.3
3	- 1	+1	+1	- 1	+1	- 1	- 1	- 1	28.9
4	+1	- 1	+1	+1	- 1	+1	- 1	- 1	27.0
5	+1	+1	- 1	+1	+1	- 1	+1	- 1	30.5
6	+1	+1	+1	- 1	+1	+1	- 1	+1	32.2
7	- 1	+1	+1	+1	- 1	+1	+1	- 1	28.8
8	- 1	- 1	+1	+1	+1	- 1	+1	+1	25.4
9	- 1	- 1	- 1	+1	+1	+1	- 1	+1	24.6
10	+1	- 1	- 1	- 1	+1	+1	+1	- 1	27.3
11	- 1	+1	- 1	- 1	- 1	+1	+1	+1	26.1
12	- 1	- 1	- 1	- 1	- 1	- 1	- 1	- 1	23.8

Note. A: reaction time (- 1) 10 min, (+1) 30 min; B: initial pH (- 1) 5.0, (+1) 9.0; C: PGK (- 1) 10.0 U/L, (+1) 30.0 U/L; D: G-6-PDH (- 1) 10.0 U/L, (+1) 30.0 U/L; E: $MgCl_2$ (- 1) 1.0 mmol/L, (+1) 3.0 mmol/L; F: temperature (- 1) 30 ℃, (- 1) 40℃; G: β-NADP (- 1) 0.5 mmol/L, (+1) 1.5 mmol/L; H: β-D(+) Glucose (- 1) 2.0 mmol/L, (+1) 10.0 mmol/L.

TABLE 2

Experimental Design Matrix and Results of CCD

Run	Variables			Activity Ratio (%)
	X_1	X_2	X_3	
1	- 1	- 1	- 1	30.0
2	+1	- 1	- 1	31.4
3	- 1	+1	- 1	31.9
4	+1	+1	- 1	32.3
5	- 1	- 1	+1	30.3
6	+1	- 1	+1	32.1
7	- 1	+1	+1	32.6
8	+1	+1	+1	33.8
9	- 1.68	0	0	31.9
10	+1.68	0	0	33.9
11	0	- 1.68	0	30.3
12	0	+1.68	0	34.5
13	0	0	-1.68	31.7
14	0	0	-1.68	31.8
15	0	0	0	33.4
16	0	0	0	33.3
17	0	0	0	33.6
18	0	0	0	33.7
19	0	0	0	33.5
20	0	0	0	33.5

Note. X_1: initial pH (- 1.68) 5.32, (- 1) 6.0, (0) 7.0, (+1) 8.0, (+1.68) 8.68; X_2 : reaction time (- 1.68) 3.2 min, (- 1) 10.0 min, (0) 20.0 min, (+1) 30.0 min, (+1.68) 36.8 min; X_3: $MgCl_2$ (- 1.68) 0.32 mmol/L, (- 1) 1.0 mmol/L, (0) 2.0 mmol/L, (+1) 3.0 mmol/L, (+1.68) 3.68 mmol/L.

used to calculate the predicted response.

$$Y=\beta_0+\Sigma\beta_i X_i+\Sigma\beta_{ii}X_i^2+\Sigma\beta_{ij}X_iX_j, \quad (1)$$

Where Y represents response variable, β_0 the interception coefficient, β_i the coefficient of linear effect, β_{ii} the coefficient of quadratic effect, and β_{ij} the coefficient of interaction effect. Each experiment was performed in duplicate with the mean value taken for analysis. A Design-Expert 6.0.5 was used for multiple

regression analysis of the experimental data. F-test was employed to evaluate the statistical significance of the quadratic polynomial. The multiple coefficients of correlation R and the determination coefficient of correlation R^2 were calculated to evaluate the performance of the regression equation.

RESULTS

Analysis of the Expression Vector 6HisT-pRSET-PGK

6HisT-pRSET-PGK was digested with NdeI + *Bam*HI, and cut into two fragments: a *gk* gene fragment (1400 bp) and a 6HisT-pRSET fragment (2922 bp) as shown in Fig. 2. The sequencing results of the recombinant plasmid, accomplished by the Sequencing Department of Invitrogen Co. Ltd, indicate that the sequence was the consensus sequence encoding *Homo sapiens* glucokinase in Genebank. As shown in Figs. 2 and. 3, the *gk* gene was inserted into the NdeI- and *Bam*HI-digested efficient prokaryotic expression vector 6HisT-pRSET-PGK, and the ORF for the *gk* gene was right.

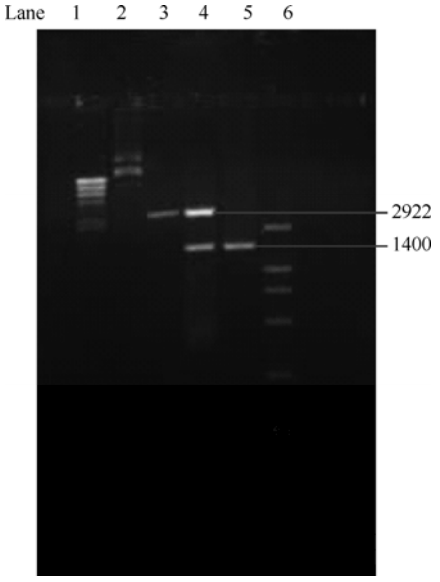

FIG. 2. 6HisT-pRSET-PGK and its digested products. Lane 1= DNA marker DL15000, lane 2= 6HisT-pRSET-PGK, lane 3= digested product of 6HisT-pRSET with NdeI-*Bam*HI, lane 4= digested product of 6HisT-pRSET-PGK with NdeI-*Bam*HI, lane 5= PCR product of *gk* gene, lane 6= DNA marker DL2000.

Expression and Purification of PGK Protein

After induction for 3 h, the expression vector 6HisT-pRSET-PGK-transfected *E. coli* BL21 could express fused protein at the final IPTG concentration

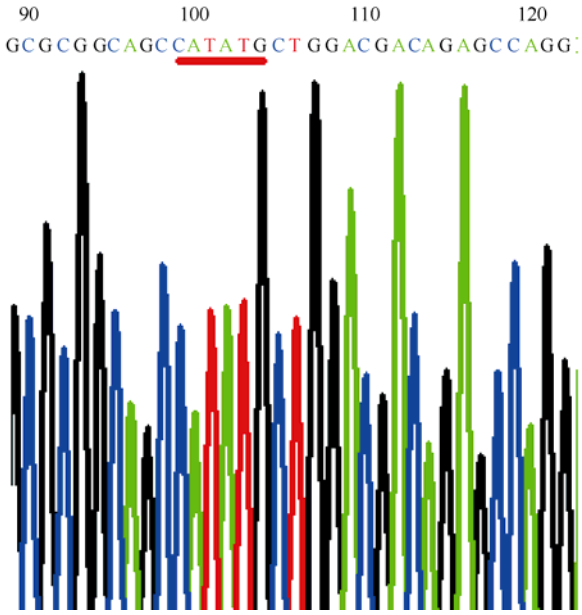

FIG. 3. 5′-end sequencing analysis map of 6HisT-pRSET-PGK recombinant plasmid.

of 0.75 mmol/L. The protein was dissoluble and its molecular weight was about 55 KDa, which was in accordance with the calculated molecular weight (Fig. 4).

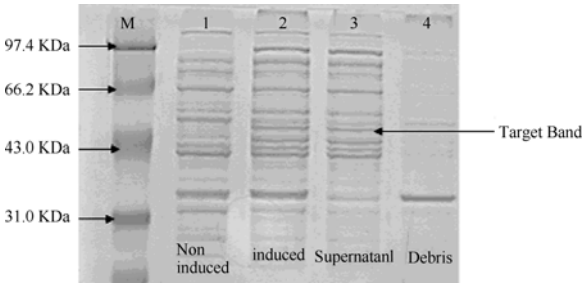

FIG. 4. SDS-PAGE analysis of the expression of recombinant protein induced by IPTG at the final concentration of 0.75 mmol/L. Lane M= protein markers (97.4 KDa, 66.2 KDa, 43.0 KDa, 31.0 KDa, 20.1 Kda, and 14.4 KDa), lane 1 = 6HisT-pRSET-PGK BL21 before IPTG induction, lane 2 = 6HisT-pRSET-PGK BL21 expression after 0.75 mmol/L IPTG induction for 3 h, lane 3= supernatant of 6HisT-pRSET-PGK BL21 expression after 0.75 mmol/L IPTG induction for 3 h, lane 4= debris of 6HisT-pRSET-PGK BL21 expression after 0.75 mmol/L IPTG inducion for 3 h.

The lysate supernatant was purified with Ni-NTA His.bind resin. A 250 mmol/L imidazole elution buffer (pH 8.0) had the optimal eluting effect and collected eluting apexes, and the final concentration of the purified protein was 2.07 mg/mL by Bradford examination. Quantity One Software analysis of 12% SDS-PAGE displayed the purity of purified PGK

approached to 100% (Fig. 5) and the specific activity of the purified PGK was 3.77 U/mg, which was diluted at 1:30 for the establishment of hypoglycemic agent screening method.

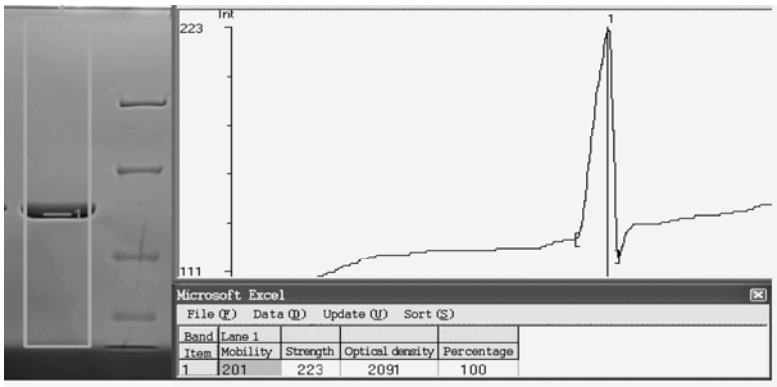

FIG. 5. Purity analysis of recombinant PGK protein with quantity one software.

Plackett-Burman Design (PBD)

At the first optimization step, a 12-run PBD was used to identify the significant factors for the activity ratio of RO-28-1675. According to the resulting effects of these eight variables on the activity ratio and the associated significant levels presented in Table 3, with the help of relative ranking, the initial pH, reaction time, and concentration of $MgCl_2$ had the most significant effects on the activity ratio of RO-28-1675. Therefore, initial pH, reaction time, and $MgCl_2$ within the tested limits were selected for further optimization.

TABLE 3

Coefficients, F Values, and Significance Levels Calculated from the Activity Ratio of GKA (RO-28-1675) in the Screening Experiments

Variables	Coefficient	F Value	Prob > F	Ranking
Reaction Time	1.07	23.45	0.0168	2*
Initial pH	1.80	66.78	0.0038	1**
PGK	0.57	6.62	0.0823	4
G-6-PDH	0.10	0.21	0.6807	8
$MgCl_2$	0.82	13.75	0.0341	3*
Temperature	0.33	2.29	0.2274	6
β-NADP	- 0.13	0.37	0.5877	7
Glucose	- 0.38	3.03	0.1802	5

Note. *Statistically significant at 95% of probability level, **statistically significant at 99% of probability level.

The significant effects of the initial pH and concentration of $MgCl_2$ were probably due to the requirement of enzyme catalysis or due to the fact that the enzyme actions need an optimal pH and $MgCl_2$ is the essential factor for PGK, which could influence the PGK structure and affect the combination of PGK, RO-28-1675 and substrate further. The PBD was proved to be a powerful tool to rapidly determine the effects of screening conditions of the activity ratio of RO-28-1675. However, the optimal conditions of screening platform could not be obtained. Further work is needed to find out this information.

Central Composite Design (CCD)

Based on the PBD, a CCD was used for further optimization. Table 2 gives the variation levels at which these conditions were used for the determination of the activity ratio of RO-28-1675. Other reaction conditions were set at their central point tested in the PB design except for temperature. A temperature of 37 ℃ was chosen to match the human body temperature. Table 2 gives the design and results of experiments carried out with the CCD. The results obtained were submitted to analysis of variance using Design-Expert 6.0.5, with the regression model given as

$$Y=33.52+0.60\,X_1+1.02\,X_2+0.25\,X_3-0.31\,X_1^2-0.49\,X_2^2-0.72\,X_3^2-0.20\,X_1X_2+0.15\,X_1X_3+0.15\,X_2X_3 \quad (2)$$

where Y is the response value, namely the activity ratio of RO-28-1675, and X_1, X_2, and X_3 are the coded levels of the initial pH, reaction time and $MgCl_2$,

respectively.

The analysis of variance of the quadratic regression model demonstrated that Eq.(2) was a highly significant model, as was evident from the Fisher's *F*-test with a very low probability value [(*P* model>*F*)=0.0001]. The model's goodness of fit was checked by determination coefficient (R^2). In this case, the value of the determination coefficient (R^2=0.9442) indicated that only 5.58% of the total variations were not explained by the model. The value of the adjusted determination coefficient [Adj(R^2)=0.8940] was also very high, supporting the high significance of the model. Among the model terms, X_1, X_2, X_2^2, and X_3^2 were significant with a probability of 99%, X_1^2 was significant with a probability of 95% (Table 4). The interaction between X_1, X_2, and X_3, however, had no significant influence.

TALBE 4

Analysis of Variance for the Fitted Quadratic Polynomial Model

Term	Coefficient	Sums of Squares	DF	F Value	Prob > F
X_1	0.60	4.88	1	26.56	0.0004**
X_2	1.02	14.07	1	76.58	0.0001**
X_3	0.25	0.83	1	4.52	0.0594
X_1^2	- 0.31	1.42	1	7.71	0.0196*
X_2^2	- 0.49	3.46	1	18.85	0.0015**
X_3^2	- 0.72	7.47	1	40.66	0.0001**
X_1X_2	- 0.20	0.32	1	1.74	0.2164
X_1X_3	0.15	0.18	1	0.98	0.3457
X_2X_3	0.15	0.18	1	0.98	0.3457

Note. X_1: initial pH, X_2: reaction time, X_3: $MgCl_2$. *statistically significant at 95% of probability level, **statistically significant at 99% of probability level.

Taking into account the pathological and physiological status in the diabetic patients *in vivo*, we kept the pH at 7.0 for the determination of the PGK activity ratio. For this reason, the model was predictive and used to generate a contour plot (Fig. 6) for the pair-wise combination of the reaction time and $MgCl_2$.

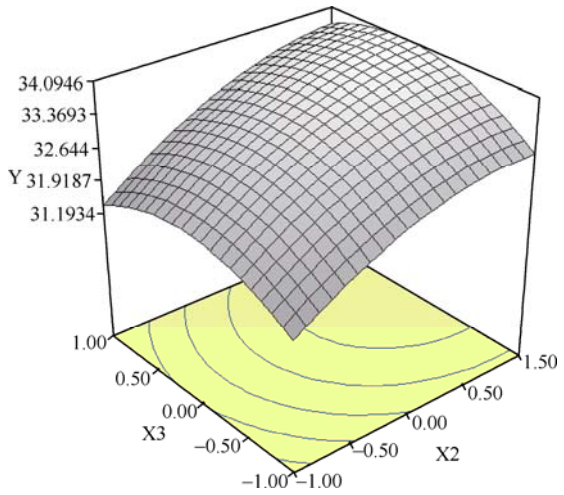

FIG. 6. Effects of reaction time (X_2) and $MgCl_2$ (X_3) and their interactive effect on the activity ratio (Y) of RO-28-1675 with the initial pH set at 7.0.

In order to make the screening work more efficient, we set the reaction time at the minimum. In light of this, the optimal levels were as follows: X_1=0 (7.0), X_2=-0.63 (13.7 min), X_3=0.11 (2.11 mmol/L) with the corresponding Y=34.1 %. Verification of the predicted values was conducted by using the optimal conditions in inoculation experiments. With 3 μmol/L RO-28-1675, the practical corresponding response was 34.8%, which corroborated the validity and effectiveness of this model.

DISCUSSION

Current therapies can not achieve adequate control of glycemia in type 2 diabetes and there is a growing need for novel drugs with improved efficacy to treat this disease[14]. GKAs represent a promising concept for the treatment of type 2 diabetes[15]. As a first step toward the development of potential hypoglycemic agents, our aim was to establish a sensitive microplate-based method to determine the activity of GKAs and provide a reliable high-throughput way for screening GKAs *in vitro*.

RSM was performed to optimize the screening system for the activity ratio of GKAs. A highly significant quadratic polynomial obtained by the CCD is extremely useful for determining the optimal levels of constituents that have significant effects on the activity ratio. For simulating the function of GKAs *in vivo*, we set the reaction temperature at 37 ℃ and the initial pH at 7.0. A 13.7 min-reaction was

selected, considering the less required time for a certain desired result. In this study, a novel reliable platform was established for screening GKAs based on human PGK protein. The reation procedure was as follows: 150 μL assay system containing 60 mmol/L Tris, 2.11 mmol/L $MgCl_2$, 4.0 mmol/L ATP, 6.0 mmol/L β-D(+) glucose, 1.0 mmol/L β-NADP, 20 U/L G-6-PDH and 20 U/L PGK, was incubated at 37 ℃ (pH 7.0) for 13.7 min and measured at 340 nm.

With this hypoglycemic agent screening method, we successfully obtained an ingredient, LACE3 extracted from *Folium Nelumbinis* by ultrasonic extraction, which has a strong PGK activated effect.

In conclusion, our model makes it possible to rapidly obtain reliable results by screening a large scale of GKAs from Chinese herbal medicines and other materials using a very small quantity of samples. In addition, human PGK can be used as a target protein in this screening platform, thus mimicking the pathological and physiological status in the diabetic patients *in vivo* and making the screening platform more reliable. Our screening method may help to pace with the development of potential hypoglycemic agents from a large number of natural products and to guide the clinical treatment of type 2 diabetes and its complications.

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REFERENCES

1. Grimsby J, Sarabu R, Corbett W L, *et al.* (2003). Allosteric activators of glucokinase: potential role in diabetes therapy. *Science* **301**(5631), 370-373.
2. Guertin K R, Grimsby J (2006). Small molecule glucokinase activators as glucose lowering agents: a new paradigm for diabetes therapy. *Curr Med Chem* **13**(15), 1839-1843.
3. Burke C V, Buettger C W, Davis E A, *et al.* (1999). Cell-biological assessment of human glucokinase mutants causing maturity-onset diabetes of the young type 2 (MODY-2) or glucokinase-linked hyperinsulinaemia (GK-HI). *Biochem J* **342**(Pt 2), 345-352.
4. Bellanné-Chantelot C, Carette C, Riveline J P, *et al.* (2007). The type and the position of HNF1A mutation modulate age at diagnosis of diabetes in patients with maturity onset diabetes of the young (MODY)-3. *Diabetes* **57**(2), 503-508.
5. Slosberg E D, Desai U J, Caplan S L , *et al*. (2001). Treatment of type 2 diabetes by adenoviral-mediated overexpression of the glucokinase regulatory protein. *Diabetes* **50**(8), 1813-1820.
6. Spraggs C, McCarthy A, McCarthy L, *et al*. (2007). Genetic variants in the epithelial sodium channel associate with oedema in type 2 diabetic patients receiving the peroxisome proliferator-activated receptor gamma agonist farglitazar. *Pharmacogenet Genomics* **17**(12), 1065-1076.
7. Park Y S, Kang S W, Lee J S, *et al.* (2002). Xylanase production in solid state fermertation by *Aspergillus niger* mutant using statistical experimental designs. *Appl Microbiol Biotechnol* **58**(6), 761-766.
8. Puri S, Beg Q K, Gupta R (2002). Optimization of alkaline protease from *Bacillus* sp. by response surface methology. *Curr Microbiol* **44**(4), 286-290.
9. Santos L O, Gonzales T A, Ubeda B T, *et al.* (2007). Influence of culture conditions on glutathione production by *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* **77**(4), 763-769.
10. Li Y, Liu Z, Cui F, *et al.* (2007). Application of Plackett-Burman experimental design and Doehlert design to evaluate nutritional requirements for xylanase production by *Alternaria mali* ND-16. *Appl Microbiol Biotechnol* **77**(2), 301-305.
11. Joseph S, David W R (2001). Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, New York.
12. Tao Y, Guermah M, Martinez E, (1997). Specific Interactions and Potential Functions of Human TAFII100. *J Biol Chem* **272**(10), 6714-6721.
13. Lin J H, Wei W L, Peng X X (2001). Mordern Biological Experiment, 1st ed. Child Hunger and Education Program Press, Beijing.
14. Tadayyon M, Smith S A (2003). Insulin sensitisation in the treatment of type 2 diabetes. *Expert Opin Investig Drugs* **12**(3), 307-324.
15. Li Y Q, Feng Z Q, Song H R, *et al.* (2006). Advances in the study of glucokinase and small molecule glucokinase activators. *Acta Pharmaccutica Sinica* **41**(5), 390-394.

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