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# Expression, identification and biological effects of a novel VPAC2-specific agonist with high stability and bioactivity

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**The development of rBAY, a recombinant peptide with the similar sequence of synthetic BAY55-9837, as a potential peptide therapeutic for type 2 diabetes is still a challenge mainly because of its poor stability in aqueous solution. To improve the peptide stability and bioactivity and investigate its biological effects for VPAC2-specific activation, RBAYL with 31 aa was designed based on sequence alignments of pituitary adenylate cyclase-activating peptides (PACAPs), vasoactive intestinal peptide (VIP), and related analogs and generated through site-directed mutagenesis. Stability analysis showed that the prepared RBAYL with three mutations (N9Q, V17L, and N28K) were much more stable than rBAY. rRBAYL (the recombinant RBAYL) was expressed and purified by gene-recombination technology via native thiol ligation on solid beads. As much as 27.7 mg rRBAYL peptide with purity over 98% was obtained from 1 L of LB medium without expensive high-performance liquid chromatography refinements. The bioactivity assay of rRBAYL showed that it displaced [$^{125}$I]PACAP38 and [$^{125}$I]VIP from VPAC2 with a half-maximal inhibitory concentration of 51 $\pm$ 6 and 50 $\pm$ 4 nM, respectively, which were similar to those of the chemically synthesized RBAYL (sRBAYL) and lower than those of Ro25-1553, an established VPAC2 agonist. rRBAYL enhances the cAMP accumulation in CHO cells expressing human VPAC2 with a half-maximal stimulatory concentration (EC50) of 0.91 nM, whereas the receptor potency of rRBAYL at human VPAC1 (EC50 of 719 nM) was only 1/790 of that at human VPAC2, and rRBAYL had no activity toward human PAC1 receptor. Western-blot assay for glucose transporter 4 (GLUT4) indicated that the rRBAYL could significantly induce GLUT4 expression more efficiently than rBAY or Ro25-1553 in adipocytes. Compared with rBAY, rRBAYL can more efficiently promote insulin release and decrease plasma glucose level in ICR mice. Our results suggested that rRBAYL is a novel recombinant VPAC2-specific agonist with high stability and bioactivity.**

*Keywords* expression; identification; recombinant agonist; VPAC2; type 2 diabetes; biological effect

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## Introduction

Pituitary adenylate cyclase-activating peptides (PACAPs), including PACAP27 and PACAP38, and vasoactive intestinal peptide (VIP) are prominent neuropeptides that are structurally related [1]. They have wide distributions in the peripheral and central nervous systems and are also secreted by immune cells [2,3]. They are implicated in a large array of physiological and pathophysiological processes related to development, growth, cancers, immune responses, circadian rhythms, the control of neuronal and endocrine cells, and functions of digestive, respiratory, reproductive, and cardiovascular systems [4]. PACAP has a specific receptor PAC1 and shares two receptors VPAC1 and VPAC2 with VIP [5]. Different biological effects are mediated by different receptors, for example, VPAC2 activation enhances glucose disposal by stimulating insulin secretion while VPAC1 activation elevates hepatic glucose output [6,7]. Because PACAP activates both VPAC1 and VPAC2, the increase in glucose production may offset the increase in insulin secretion. Therefore, clinical treatment of diabetes requires a VPAC2-specific agonist which would enhance pancreatic β cell insulin release without causing increased glucose production by the liver and would thereby lead to increased glucose disposal.

A number of glucagon-like peptide-1 (GLP-1) receptor agonists promote glucose-dependent insulin secretion and have demonstrated glucose-lowering activity in clinical studies [8]. However, nausea and vomiting are common side effects associated with the inhibition of gastrointestinal motility by GLP-1 [9]. Unlike GLP-1 receptor agonists, a VPAC2-selective agonist has the potential to lower blood glucose without causing nausea and vomiting. In addition, VPAC receptor agonists have been reported to have

potential anti-inflammatory activity that could be beneficial in treating type 2 diabetes [10].

Currently, several VPAC2-selective agonists have been developed and hoped to be the peptide therapeutics for type 2 diabetes. Ro25-1553 and hexanoyl-VIP (C6-VIP) produced by chemical synthesis were two VIP derivatives with high preference for VPAC2 but recognized different receptor domains [11,12]. But chemical synthesis of a peptide of about 30 amino acids residues is rather time-consuming and costly. RMROM and RMBAY are PACAP derivatives on VPAC2-selective activation and have been produced by DNA recombination technology and purified by high-performance liquid chromatography (HPLC) [13,14]. rBAY, the recombinant peptides with the similar amino acids sequence as BAY55-9837, are highly selective VPAC2 receptor agonists and have been produced by high throughput mutagenesis of VIP and PACAP [15]. Especially, the development and production for RMBAY and rBAY will be more attractive. However, their poor peptide stability in aqueous solution was the main limitation for their clinical applications. Furthermore, the problems of yield, purification process and product bioactivity are still big challenges in recombinant peptide production [16].

In this study, RBAYL with 31 aa was designed and generated through site-directed mutagenesis based on sequence alignments of PACAP, VIP, and related analogs. To achieve highly efficient production of rRBAYL, the peptide was expressed and purified by gene-recombination technology via native thiol ligation and factor Xa cleavage on solid beads [17,18]. The high yield and purity (>98%) of rRBAYL can be achieved by the current method without expensive HPLC refinements. The recombinant RBAYL (i.e. rRBAYL) peptides (N9Q, V17L, and N28K) were more stable and could promote the insulin release and decrease plasma glucose level in ICR mice with higher bioactivity than it rBAY. rRBAYL can hopefully become a novel peptide therapeutic against type 2 diabetes and the production method could also be applicable to other recombinant pharmaceutical peptides.

## Materials and Methods

### Materials

ICR mice were supplied by Chinese academy of medical sciences (Beijing, China) and were housed at 22°C on a 12:12 h light–dark cycle. Ni–NTA beads were from Qiagen (Suite, USA). Affi-Gel 10 beads were supplied by Bio-Rad laboratories (Hercules, USA). Alanine ethyl thioester [$H_2NCH(CH_3)C(O)SCH_2CH_3$] was purchased from Novabiochem (La Jolla, USA). All the restriction enzymes were purchased from New England Biolabs (Ipswich, USA). The T4 DNA ligase was obtained from Takara Biotechnology (Dalian, China). Synthetic peptides were purchased from Sinoasis Pharmaceuticals (Guangzhou, China). Primer synthesis and DNA sequencing were performed by Invitrogen Company Guangzhou Branch (Guangzhou, China). pCFH vector, human VPAC1-CHO, human VPAC2-CHO, and human PAC1-CHO cell lines were constructed previously by our laboratory [13]. 3T3-L1 adipocytes were obtained from Department of Biology, Wuhan University (Wuhan, China). Cyclic AMP enzyme immunoassay kits were purchased from Cayman Chemical Company (Ann Arbor, USA).

### Preparation of thioester solid beads

Affi-Gel 10 was used as the solid matrix. The coupling of alanine ethyl thioester to Affi-Gel 10 was performed under anhydrous conditions according to a standard procedure provided by the supplier. Briefly, the amino acid thioester was dissolved in DMSO and mixed with certain amount of the beads and the mixture was gently stirred for 5 h at room temperature. At the end of the reaction, 0.2 ml ethanolamine (1 M in DMSO) per ml gel was added to block any active ester on the beads.

### Expression of fusion protein CFH-Xa-RBAYL

Fusion proteins containing RBAYL peptides were prepared with a recombinant DNA approach. Briefly, RBAYL gene was designed by use of the codon preference of *E. coli* to ensure the high expression in *E. coli*. The gene was synthesized and amplified in two steps as described previously [13] using four oligonucleotide primers: $F_1$: 5′-NNNNNGAATTCATTGAAGGTCGTCATAGCGATGCGGTGTTTACCGATCAGTATACCCGTCTGCGTAAACAG-3′, containing an *Eco*RI site (underlined); $F_2$: 5′-CTGGCGGCGAAAAAATATCTGCAGAGCATTAAAAACAAACGTTAT-3′; $R_2$: 5′-CGCCAGCTGTTTACGCAGACGGGTATACTGATCGGTAAACACCGCATCGCTATGCA-3′; and $R_1$: 5′-NNNNNNGGATCCTTAATAACGTTTTTTTTTCAGGCTCTGCAGATATTTTTTCGC-3′, containing a *Bam*HI site (underlined). 'N' represents the protecting base. After PCR products were purified by the Qiagen PCR clean-up kit (Qiagen) and double digested with *Eco*RI and *Bam*HI, the DNA inserts were ligated into the predigested expression vector pCFH-MCS. In the pCFH-MCS expression vector, one TGC codon encoding cysteine was at the 5′ end of *CFH* gene. The resultant expression constructs named as pCFH-RBAYL with *RBAYL* gene were confirmed by DNA sequencing using the T7 promoter as the sequencing primer (**Fig. 1**).

pCFH-RBAYL expression vector was transformed into the *E. coli* BL21 (DE3) host strain for protein expression with the optimized procedure [19]. Briefly, the cells were grown at 37°C to a density of $OD_{600} = 0.8$ and induced by adding IPTG to a final concentration of 0.6 mM. The induced

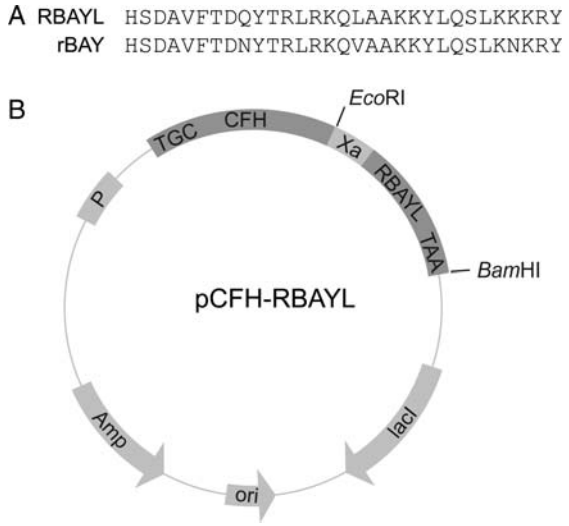


**Figure 1 An expression vector was constructed to express RBAYL peptide** (A) The amino acid sequence of RBAYL and rBAY. (B) The map of the constructed peptide expression vector. One unique protein domain, CFH, was used as the carrier protein without Ile-Glu-Gly-Arg in the sequence. P denoted the T7 promoter. A His-tag with six histidines is placed at the C-terminal of the CFH carrier protein to simplify the protein purification. One single cysteine residue is placed at the N-terminal of the CFH carrier protein to facilitate native thiol ligation. Xa denotes factor Xa cleavage site: Ile-Glu-Gly-Arg.

cells were incubated for 6 h at 37°C and collected by centrifugation at 10621 *g* for 20 min. SDS-PAGE was used to identify the expression of the fusion protein [20]. The cell pellet was resuspended in a lysis buffer containing 6 M urea, 20 mM Tris–HCl (pH 8.0), 100 mM NaCl, and 10 mM TCEP (Tris[2-carboxyethyl]phosphine TFE,2,2,2-triXuoroethanol) by gentle shaking for 20 min and then disrupted with JN-3000 PLUS low-temperature ultra-high-pressure continuous flow cell crusher (JNBIO, Guangzhou, China). The lysate was then centrifuged at 10621 *g* at 4°C for 30 min and the supernatant was subjected to purification by Ni–NTA affinity chromatography under a denaturing condition. After the fusion protein was eluted from the column with the lysis buffer containing 250 mM imidazole. The eluate was directly used for the preparation of the target peptide.

### Preparation of target peptide rRBAYL

The thioester beads prepared from the aforementioned procedure were put into glass chromatographic column (Bio-Rad, Hercules, USA) and mixed with the fusion protein eluate in the ratio of 1:8 (*v/v*). Four percent (*v/v*) benzymercaptane and 2% (*w/v*) MESNA were added. The column was shaken to equilibrate the thiol additives in darkness at room temperature. After the reaction was complete, the beads were extensively washed with the cleavage solution containing 1 M NaCl, 500 mM Tris–HCl, 50 mM $CaCl_2$ (pH 8.0), and suspended in two gel-bed volume of the same solution. Factor Xa was added to the suspension in a ratio of 10 units per mg fusion protein. All the cleavage reactions were performed at 20°C in dark for 16 h and monitored with HPLC. Factor Xa in eluate was removed with Xarrest Agarose (Novagen, Madison, USA), and the target peptide was recovered by spin filtration. The target peptide, the recombinant RBAYL (i.e. rRBAYL) was then desalted by using a 4.6 mm × 150 mm 300 SB-C18 Sep-Pak column (Agilent Technologies, Santa Clara, USA) and dried by lyophilization.

### Characterization of rRBAYL by HPLC and MS

rRBAYL, the target peptide prepared from either desalting or lyophilized powder form were dissolved in 2% acetonitrile, 0.1% TFA. Samples were analyzed by reverse phase HPLC system using 4.6 mm × 150 mm 300 SB-C18 Sep-Pak column (Agilent Technologies) and eluted with increasing concentration of acetonitrile from 5% to 60% for 50 min at 1 ml/min. Prepared rRBAYL at a final concentration of 1 mg/ml in 45% acetonitrile containing 0.1% TFA was analyzed by 4000 Q TRAP electrospray ionization mass spectrometry (ESI-MS) (Applied Biosystems, Foster City, USA). Peptide concentrations were determined by comparing the $OD_{280}$ values of peptide stock solutions in the assay buffer with the predicted extinction coefficient [21].

### Stability determination

rRBAYL or rBAY at a final concentration of 1 mg/ml in 20 mM sodium phosphate buffer (pH 8.0) containing 150 mM sodium chloride were incubated at 37°C. At different time points, samples were collected and analyzed by LC-MS, a rapid and sensitive method to detect degradation of polypeptide in these formulations. A 2-μl sample was injected into HPLC-ESI-MS system containing 1.0 mm × 150 mm 300 SB-C18 Sep-Pak column (Agilent Technologies) and analyzed under the condition of increasing concentration of acetonitrile from 2 to 55% for 50 min at 0.05 ml/min by HPLC (Agilent Technologies)-ESI-MS (Applied Biosystems) system.

### Receptor competition-binding assay

The potential of rRBAYL to displace [$^{125}$I]PACAP38 and [$^{125}$I]VIP by binding competitively to the human VPAC2 receptor was examined in VPAC2-CHO cell membrane prepared through previous methods [14]. Briefly, 10 μg of membrane was incubated with 0.1 nM [$^{125}$I]PACAP38 (Phoenix Pharmaceuticals, Mountain View, USA) or [$^{125}$I]VIP (PerkinElmer Life and Analytical Sciences, Boston, USA) in the presence of increasing concentrations of RBAYL peptide, in a total volume of 100 μl of 20 mM HEPES (pH 7.4) containing 150 mM NaCl, 0.5% BSA, 2 mM $MgCl_2$, and 0.1 mg/ml bacitracin at 37°C. After 20 min, the membrane was collected on GF/C filters pretreated with 0.1%

polyethylenimine. The filters were washed with cold 25 mM $Na_3PO_4$ containing 1% BSA and counted on a gamma counter. Nonspecific binding was defined as the residual binding in the presence of 1 μM recombinant PACAP38 (i.e. rPACAP38) or VIP and was always less than 20% of the total binding. The assay of synthetic RBAYL (i.e. sRBAYL), PACAP38, VIP, and Ro25-1553 were taken as the positive controls. [$K^{15}$,$R^{16}$,$L^{27}$]VIP(1−7)/GRF(8−27), a VPAC1-specific agonist, was used as the negative control in the receptor binding assay [22]. Each assay was performed at least three times.

### Assay of cAMP accumulation induced by rRBAYL and VIP

Human PACAP receptor-transfected cells, VPAC1-CHO, VPAC2-CHO, and PAC1-CHO cells, cultured in the DMEM at 37°C were scraped off with rubber policeman and washed with PBS twice. The density of the cells was adjusted to $2 \times 10^6$ cells/ml. rRBAYL or VIP was added to 500 μl cell suspension, and the working concentrations of the peptide were ranged from $1 \times 10^{-12}$ to $1 \times 10^{-5}$ M. The mixtures were incubated at 37°C for 5 min and then two volumes of 0.2 M HCl was added and incubated at room temperature for 20 min. Cells were lysed by pipetting up and down until the suspension was homogeneous. The precipitate was removed by centrifugation at 1200 rpm for 10 min, and the supernatant was transferred into test tube and cAMP concentrations were measured by using the cyclic AMP enzyme immunoassay kit.

### Western blot analysis of GLUT4 induced by rRBAYL

Cell culture and induction for 3T3-L1 adipocytes were performed as described previously [23]. Differentiated 3T3-L1 adipocytes were cultured for 48 h in medium containing 5 nM of rRBAYL, sRBAYL, rBAY, VIP, or Ro25-1553, respectively, and then the total protein was extracted. After the total protein was separated by 12% SDS-PAGE and transferred onto PVDF membranes (Immobilon P, Millipore, USA), the membranes were incubated with the anti-GLUT4 antibody (Santa Cruz Biotechnology, Santa Cruz, USA) at 1:400 dilution in TBST containing 5% skim milk for 2 h at room temperature. The sheep-anti-mouse HRP-IgG (BioFX Laboratories, Owings Mills, USA) (1:7000) was used as the second antibody. Protein bands were visualized by using an ECL kit (Santa Cruz Biotechnology) and densitometric analysis of the results of western blot was performed with Scion image software [24].

### *In vivo* biological effects of rRBAYL in ICR mice

Twenty-four male ICR mice weighing 26−30 g were housed at 22°C on a 12:12 h light−dark cycle. ICR mice fasted overnight (12 h) and were randomly divided into three groups according to their weight (eight mice/ group). Glucose (1.8 mmol/kg) with or without the prepared rRBAYL (50 ng/kg), which is dissolved in the normal saline was intraperitoneally injected into the ICR mice. The experimental groups with the same dose or volume of sRBAYL, rBAY, VIP, and Ro25-1553 were as positive controls and the groups with normal saline as blank control. Blood samples were collected from the tail vein before injection and at 10 min after the injection. The plasma glucose levels were determined using OneTouch Ultra Meter (Johnson & Johnson, Johnson, USA) and the plasma insulin were measured using RIA kit (Linco Research, Charles, USA) in the first affiliated hospital of Jinan University.

## Results

### Expression and purification of rRBAYL

In the expressed fusion protein (i.e. CFH-Xa-RBAYL in **Fig. 2**), RBAYL peptide was fused with the fusion carrier protein (i.e. CFH) through an individual factor Xa cleavage site—Ile-Glu-Gly-Arg. The fusion proteins purified with Ni−NTA His-tag affinity chromatographic column under denaturing condition were directly used for the thiol ligation to generate the beads coupled with fusion protein as described in **Fig. 2(A)**. The addition of benzymercaptane (4%, *v/v*) and MESNA (2%, *w/v*) to reaction mixtures was confirmed to enhance the thiol ligation. The ligation was monitored by measuring the decrease of protein concentration in the reaction mixtures. **Fig. 2(B)** shows a time course of thiol ligation for both Affi-Gel 10 beads and the fusion protein—CFH-Xa-RBAYL. The coupling reaction was found to be complete in 5 h.

The fusion protein, CFH-Xa-RBAYL, was cleaved on Affi-Gel 10 solid beads by factor Xa. The cleavage reactions were performed with gentle shaking at 20°C in dark and monitored with analytical HPLC method. The HPLC peak area that reflected the concentration of RBAYL in solution reached a plateau after 16 h and the cleavage efficiency was over 98%. In order to remove the factor Xa in reaction mixtures, Xarrest agarose was used for factor Xa capture with the approach as described in Manual. When using 1× cleavage/capture buffer, more than 99% factor Xa can be efficiently removed with Xarrest agarose in a ratio of 50 μl settled resin (100 μl of the 50% slurry) per 4 units factor Xa in a 5 min incubation. The capture efficiency was analyzed by LC-MS and over 99% factor Xa could be removed from the mixtures. The recombinant RBAYL peptide was obtained after simple desalting with a 4.6 mm × 150 mm 300 SB-C18 Sep-Pak column and lyophilization.

Through the current strategy, the expression of the fusion protein reached 252 mg and as much as 27.7 mg recombinant RBAYL with purity over 98% [**Fig. 3(B)**] could be obtained from 1 L of the LB culture medium (**Table 1**).

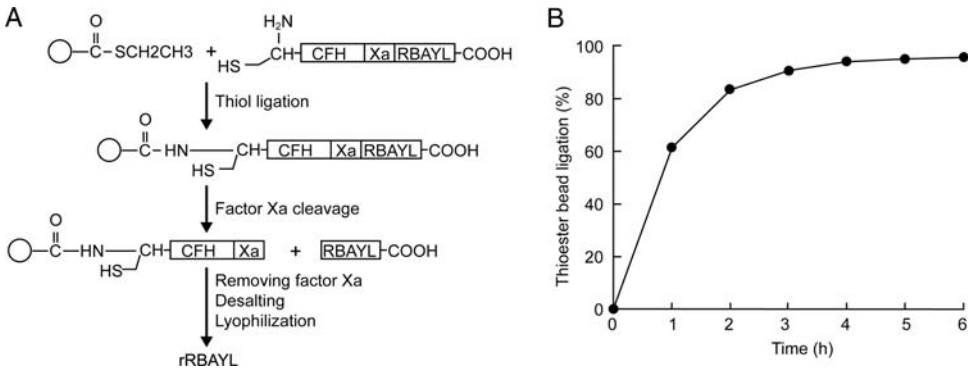


**Figure 2 Preparation of recombinant RBAYL peptide by factor Xa cleaving Affi-Gel 10 beads-attached fusion protein** (A) The preparation procedure of RBAYL in the current work. (B) More than 95% of thiol ligation of fusion protein containing the recombinant RBAYL to thioester solid beads can be achieved within 5 h. ○, Affi-Gel 10 beads; Xa, factor Xa cleavage site: Ile-Glu-Gly-Arg.

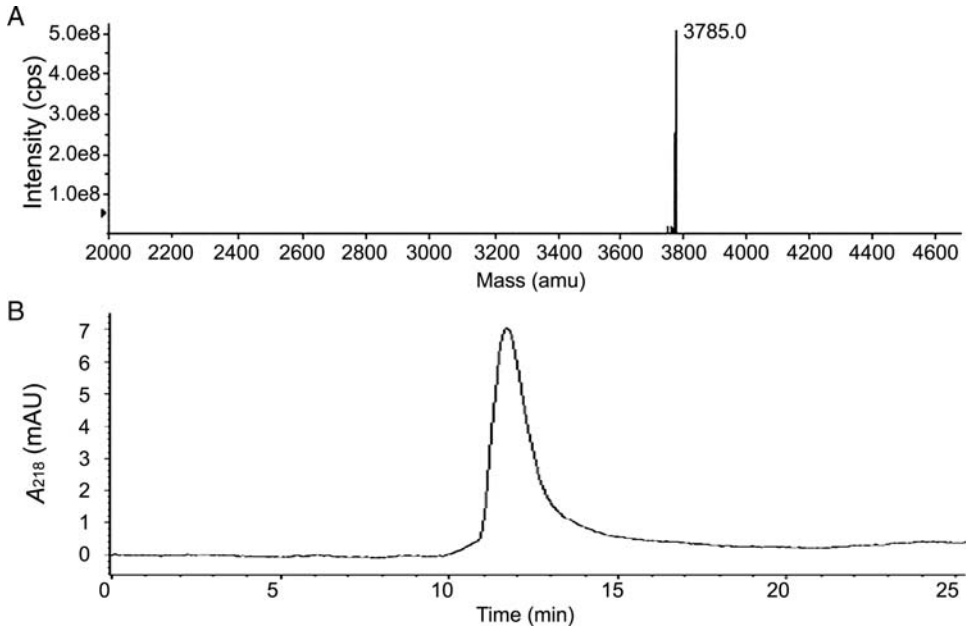


**Figure 3 Analysis of prepared recombinant RBAYL by HPLC and MS** (A) Electrospray ionization TOF-MS of the prepared recombinant RBAYL. (B) The HPLC analysis of the purified recombinant RBAYL.

**Table 1 Preparation of recombinant RBAYL peptide from 1 L culture**

| Purification step | Yield (mg) | Purity[a] |
|---|---|---|
| His-tag affinity purification[b] | 252 ± 6.3 | >98% |
| rRBAYL yield after cleavage | 30.2 ± 4.0 | ND |
| rRBAYL yield after desalting | 27.7 ± 2.1 | >98% |

ND, not determined. All the data are presented as mean ± SD of three individual experiments. [a]Purity of protein was determined by HPLC method. [b]After His-tag affinity purification.

## Characterization of prepared rRBAYL by HPLC and MS

RBAYL prepared through the current strategy (i.e. rRBAYL) was identified by ESI-MS. **Figure 3(A)** showed the result that the molecular weight of rRBAYL from ESI-MS was 3785.0 Da, which was consistent with the theoretical value (MW: 3785.4 Da). The purity of prepared rRBAYL was determined by the analytical HPLC method. **Figure 3(B)** showed that it was over 98%.

## Peptide stability increased by mutations

The newly prepared rRBAYL was also tested together with rBAY for stability at 37°C in 20 mM sodium phosphate buffer (pH 8.0) containing 150 mM sodium chloride. After 4 weeks at 37°C, the main peptide peak for rBAY was remarkably diminished and the slower migrating peak emerged, probably as a result of peptide degradation. On the other hand, rRBAYL exhibited dramatic improvement in stability, losing only 8.5% (normalized by the percentage purity at zero time) of the main peak during the 4-week incubation at 37°C. The stability data in 4 weeks

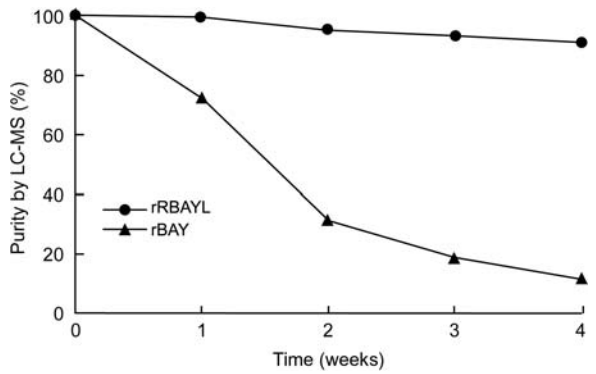


**Figure 4 Stability analysis of peptides at 1 mg/ml in aqueous solution during incubation at 37°C**

showed that rRBAYL mutants were much more stable than rBAY (**Fig. 4**).

### rRBAYL competitively binding to VPAC2 receptor

Competitive receptor binding was measured for the recombinant RBAYL peptide (i.e. rRBAYL), the synthetic RBAYL (i.e. sRBAYL) peptide, rPACAP38, VIP, Ro25-1553, and [$K^{15}$,$R^{16}$,$L^{27}$]VIP(1−7)/GRF(8−27) peptide using plasma membranes derived from VPAC2-CHO cells expressing VPAC2 receptor. rRBAYL competitively displaced [$^{125}$I]PACAP38 from VPAC2 with a half-maximal inhibitory concentration (IC50) of 51 ± 6 nM, which was similar to sRBAYL with IC50 of 49 ± 7 nM, and the IC50 for rPACAP, VIP, and Ro25-1553 at human VPAC2 were 17 ± 6, 20 ± 4, and 61 ± 6 nM, respectively [**Fig. 5(A)**]. rRBAYL competitively displaced [$^{125}$I]VIP from VPAC2 with an IC50 of 50 ± 4 nM, which was similar to sRBAYL with IC50 of 47 ± 5 nM, and the IC50 for rPACAP, VIP, and Ro25-1553 at human VPAC2 were 19 ± 5, 18 ± 4, and 63 ± 5 nM, respectively [**Fig. 5(B)**]. Whereas the IC50 for [$K^{15}$,$R^{16}$,$L^{27}$]VIP(1−7)/GRF(8−27), an established human VPAC1-specific agonist, at human VPAC2 was over 20 μM. The results showed that rRBAYL could displace [$^{125}$I]PACAP38 and [$^{125}$I]VIP by competitively binding to human VPAC2 receptor in VPAC2-CHO cells and the IC50 of rRBAYL for [$^{125}$I]VIP was slightly lower than that for [$^{125}$I]PACAP38. In two competition receptor-binding experiments, the IC50 of rRBAYL was significantly lower than that of Ro25-1553, the established VPAC2-specific agonist.

### rRBAYL enhances cAMP accumulation as a VPAC2-specific agonist

The accumulation of cAMP in human PACAP receptor-transfected cells (VPAC1-CHO, VPAC2-CHO, and PAC1-CHO cells) was used as an index of the agonist activity. rRBAYL was a potent agonist for the VPAC2 receptor with a half-maximal stimulatory concentration (EC50) of 0.91 nM. However, the receptor potency of rRBAYL at human VPAC1 [EC50 of 719 nM, **Fig. 6(A)**] was only 1/790 of that at human VPAC2, and rRBAYL had no activity toward human PAC1 receptor. cAMP accumulations induced by rRBAYL or sRBAYL in VPAC1-CHO was similar to those in VPAC2-CHO cells (sRBAYL with EC50 of 713 and 0.95 nM at human VPAC1 and VPAC2, respectively). Howerver, VIP was a potent agonist at human VPAC1 and VPAC2 receptor with an EC50 of 0.87 and 0.89 nM, respectively, and the EC50 for VIP at human PAC1 receptor was 671 nM [**Fig. 6(B)**]. These results showed that rRBAYL was a VPAC2-spcific agonist with high potency and bioactivity, whereas VIP could bind human VPAC1 and VPAC2 receptor with high affinity and bind human PAC1 receptor with low affinity.

### rRBAYL enhances the expression of GLUT4 in differentiated 3T3-L1 adipocytes

The expression of GLUT4, an important rate-limiting factor for the glucose transport, was significantly increased in differentiated 3T3-L1 adipocytes treated with rRBAYL or sRBAYL. Compared with the blank control group without rRBAYL in the culture medium, GLUT4 protein



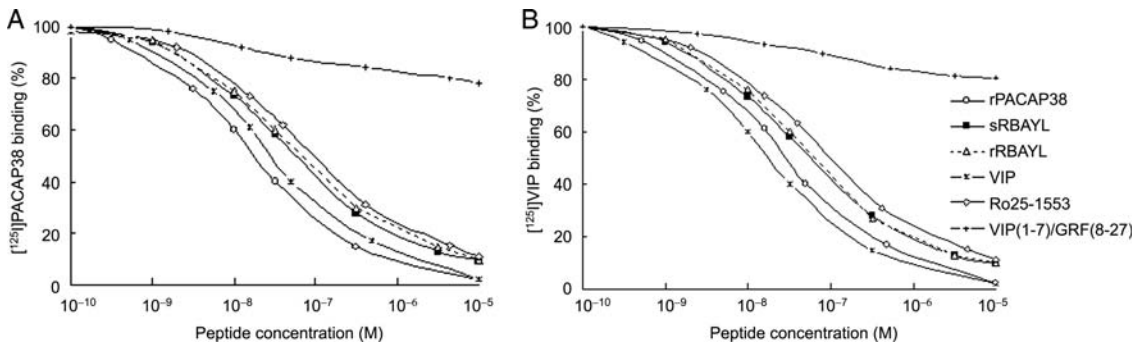


**Figure 5 Displacement of [$^{125}$I]PACAP38 (A) or [$^{125}$I]VIP (B) by rRBAYL, sRBAYL, rPACAP38, VIP, Ro25-1553, and VIP(1−7)/GRF(8−27) in membranes purified from CHO cells expressing human VPAC2** The results are expressed as percentage of maximum binding to [$^{125}$I]PACAP38 or [$^{125}$I]VIP.

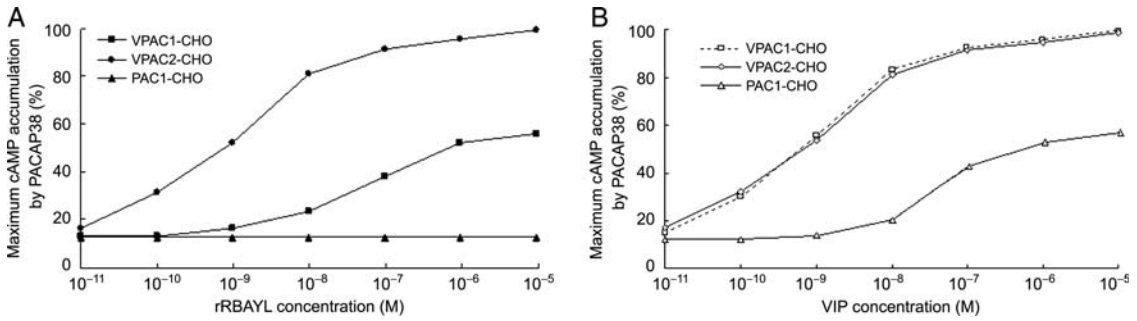


**Figure 6 Induced cAMP accumulation by RBAYL (A) or VIP (B) in CHO-VPAC2, CHO-VPAC1, and CHO-PAC1 cells** Results are expressed as the percentage of maximum cAMP accumulation by PACAP38. Data are mean of three separate experiments.

was increased by 1.3 folds in treated cells with rRBAYL. The effects of increasing GLUT4 expression by rRBAYL, sRBAYL, or VIP were similar and stronger than rBAY and Ro25-1553 (**Fig. 7**). These results showed that rRBAYL might significantly induce the GLUT4 expression and provide an important transporter for glucose uptake into tissue.

### rRBAYL promotes insulin release and glucose decrease in ICR mice

As shown in **Table 2**, compared with normal saline group, recombinant RBAYL (50 ng/kg) obviously promoted the insulin release and decreased the level of plasma glucose after intraperitoneal injection with high concentration of glucose (1.8 mmol/kg) in ICR mice. Furthermore, the results showed that the bioactivity of rRBAYL was higher than that of its recombinant analog, rBAY.

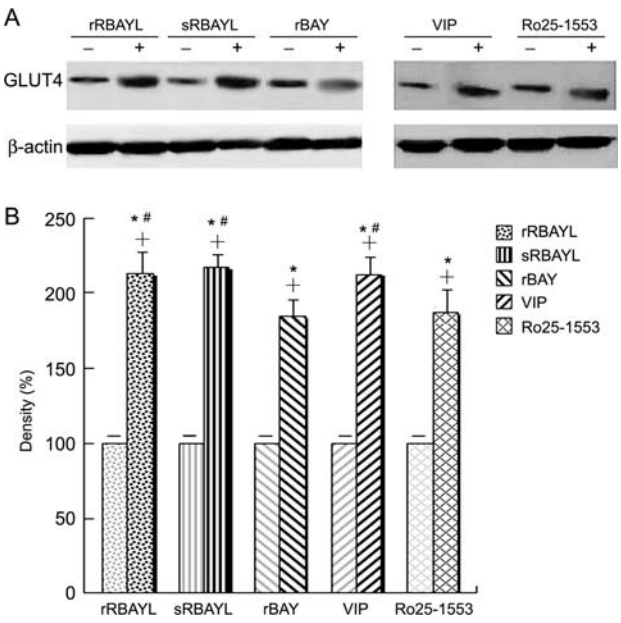


**Figure 7 Expression of GLUT4 in differentiated 3T3-L1 adipocytes cultured with or without RBAYL (A) Effect of rRBAYL, sRBAYL, rBAY, VIP, and Ro25-1553 on the expression level of GLUT4 by western blot** (B) Results of changes of GLUT4 level by densitometric analysis. −, control group; +, positive group; $^{*}P < 0.01$ compared with control group; $^{\#}P < 0.05$ compared with rBAY positive group.

**Table 2 Biological effects of rRBAYL on insulin and glucose in ICR mice**

| Group | rRBAYL group | rBAY group | Normal saline group |
|---|---|---|---|
| Plasma glucose (mM) | $11.79 \pm 2.21^{a,b}$ | $13.32 \pm 3.01^{a}$ | $20.76 \pm 3.52$ |
| Plasma insulin (mIU/L) | $49.85 \pm 3.03^{a,b}$ | $46.43 \pm 2.10^{a}$ | $32.97 \pm 2.19$ |

Data are presented as means ± SD, $n = 8$. $^{a}P < 0.01$, rRBAYL group or rBAY group vs normal saline group; $^{b}P < 0.05$, rRBAYL group vs rBAY group.

## Discussion

Glucose-dependent insulin secretion has long been considered a desirable approach to the treatment of type 2 diabetes because it can reduce blood glucose levels without the risk of hypoglycemia. Though GLP-1 receptor activation leads to glucose-dependent insulin secretion from pancreatic β cells [8], nausea and vomiting may arise with GLP-1 receptor activation [9]. VPAC2-specific agonists such as rBAY or BAY55-9837 have also been demonstrated to induce insulin secretion from β cells in a glucose-dependent manner [6], yet did not cause gastrointestinal side effects associated with VPAC1 [15]. However, the peptide instability resulting from degradation was the main problem for rBAY or BAY55-9837 as an insulin secretagogue for the treatment of type 2 diabetes. Studies on two mutations, M17V and N24Q, have been carried out in rBAY or BAY55-9837 in order to reduce the potential peptide instability due to either oxidation or deamidation, respectively. But the two peptides were still instable in liquid formulation over 2 days, which is unlikely to be due to self-hydrolysis. Based on the analysis of amino acid composition and some experiment results (data not shown), we speculate that deamidation at two asparagine residues,

N9 and N28, especially N9, in BAY55-9837 or rBAY was the main reason resulting in peptide instability. So two mutations, N9Q and N28K, as well as another mutation of V17L previously applied in RMROM and RMBAY, were simultaneously introduced into the peptide sequence by gene recombination. The recombinant peptide, rRBAYL, whose peptide sequence includes three mutations, displayed no significant degradation when stored at 37°C for 4 weeks, while rBAY showed about 90% degradation in same conditions.

Compared with two previously studied recombinant VPAC2 agonists, RMROM and RMBAY [11,13], in addition to the two major mutations (N9Q and N28K), there was no methionine at the N-terminus of the recombinant RBAYL, which may maintain the high flexibility of the N-terminal region and avoid N-terminal oxidation. The conservative N-terminal sequence H-S-D and its high flexibility in VPAC2-selective agonists were very important for VPAC2 binding and activation. PACAP, VIP, Ro25-1553, BAY55-9837, rBAY, and hexanoyl-VIP (C6-VIP) shared the same N-terminal sequence [12,14].

To conveniently achieve highly efficient expression and purification of the recombinant VPAC2-specific agonist, rRBAYL, as potential therapies for type 2 diabetes [15], in this report, we developed a combination strategy—native thiol ligation and factor Xa cleavage on solid beads without expensive HPLC step to produce RBAYL with high yield and purity. The target peptide, rRBAYL, was expressed together with a small and highly expressed carrier protein, and the peptide release was achieved by a specific factor Xa cleavage. Compared with some protein expression systems, in general, the expression yield of the purified peptides is similar to, or lower than, that used with the pCFH protein expression system [17]. In the current strategy, uncoupled fusion proteins with thioester solid beads and impurities can be efficiently removed by extensive washing. Furthermore, the use of the solid support can avoid contaminations caused by incomplete cleavages. Thus, the expression level of the fusion protein reached 252 and 27.7 mg rRBAYL peptide with purity over 98% from 1 l of induced LB medium culture. The yield of rRBAYL by current strategy was about 3 fold higher than by previously normal methods. The new strategy could also be applicable to other recombinant peptide expression systems no matter whether the fusion proteins are soluble or not. Currently, peptide drugs have gradually become a potent tool for treating some important diseases, such as diabetes, intestinal cancer, and cerebral embolism [25–27]. Some panned peptides have also been used to diagnose some diseases or act as vaccines [28–30]. The strategy should provide a useful method for facilitating the production or improving the yield and purity of recombinant pharmaceutical peptides.

Through the current strategy, the prepared rRBAYL had similar binding affinity for VPAC2 receptor and similar bioactivity of inducing cAMP accumulation and increasing GLUT4 expression to the chemically synthetic RBAYL (i.e. sRBAYL). As a VPAC2-spcific agonist, rRBAYL had higher potency and bioactivity than rBAY and Ro25-1553. The rRBAYL can significantly induce the expression of GLUT4 in differentiated 3T3-L1 adipocytes and can obviously promote the insulin release and decrease the level of plasma glucose in ICR mice. This may be due to the fact that rRBAYL potently stimulated cAMP generation, and as an important second messenger, cAMP increased the GLUT4 and insulin gene transcription and expression [31,32]. Thus, *in vivo* increase of GLUT4 expression may provide more transporter, one of the necessary conditions for glucose uptake into adipose cells and muscular tissue [33–35]. In addition, the insulin expression and secretion were effectively promoted by rRBAYL in a glucose-dependent fashion, so blood glucose could be quickly restored to normal physiological level in type 2 diabetes. Accordingly, the insulin sensitivity could be improved and insulin resistance could be decreased by the series of biological effects of rRBAYL. This work not only provided a useful method for conveniently improving yield and purity of recombinant pharmaceutical peptides, but also identified a novel recombinant VPAC2-specific agonist with high stability and bioactivity, rRBAYL, and preliminarily revealed its biological effects and mechanism against type 2 diabetes. This study will further facilitate the development of recombinant VPAC2-specific agonists such as rBAY as a potential peptide therapeutic strategy for the treatment of type 2 diabetes.

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