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Construction of a Fusion Enzyme System by Gene Splicing as a New Molecular Recognition Element for a Sequence Biosensor

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A bifunctional fusion enzyme system constructed by gene splicing is proposed as a new model to develop sequence biosensors, taking maltose biosensor as an example. The cDNA fragment of *Aspergillus niger* glucoamylase (E.C 3.2.1.3, GA) was fused to the 3′ end of *Aspergillus niger* glucose oxidase (E.C 1.1.3.4, GOD) gene with the insertion of a flexible linker peptide [-(Ser-Gly)$_5$-] coding sequence. The fusion gene was cloned into the vector pPIC9 and expressed in *Pichia pastoris* GS115 under the control of the AOX1 promoter. It was found that a bifunctional hybrid protein with a molecular weight of 430 kDa was secreted after induction with methanol. The fusion enzyme GOD-(Ser-Gly)$_5$-GA (GLG) was purified using Q Sepharose Fast Flow ion-exchang chromatography. Kinetic analysis demonstrated that GLG retained the typical kinetic properties of both GA and GOD. After being immobilized on an aminosilanized glass slide through covalent bonding by glutaraldehyde, GLG showed much higher sequential catalytic efficiency than the mixture of separately expressed GA and GOD (GA/GOD). Maltose biosensors were fabricated with GLG and GA/GOD, respectively. The performance characteristics of the maltose biosensor with respect to reproducibility, signal level, and linearity were effectively improved by using the fusion enzyme. Our findings offer a basis for the development of other sequence biosensors.

INTRODUCTION

Sequence biosensors, which involve two or more enzymes for sequential catalysis, are widely used in the determination of biochemical substances, such as disaccharides (*1*−*4*), starch (*5*), inosine (*6*), total cholesterol (*7*), etc. Upon review of the abstract books of the World Congress on Biosensors of the past decade, it is found that about 30% of the reported biosensors are sequence biosensors. For a two-enzyme sequence biosensor, for example, the reaction in the enzyme membrane could be described as follows:

$$S \xrightarrow[\text{step I}]{E1} D \xrightarrow[\text{step II}]{E2} P + B$$

The substrate S is catalyzed by the first enzyme E1 to produce the intermediate product D in step I. D then serves as the substrate of the second enzyme E2 in step II, giving the final product P and byproduct B, which could be a chemical substance or a physical signal. S is then determined by measuring P or B through an appropriate transducer. However, the performance of the sequence biosensors is normally not as good as the sole enzyme biosensors. The main reason is that the existing immobilization methods, cross-linking or covalent bonding in most cases, provide no warranty to prepare the high quality multiple enzyme membrane that is the key element of a biosensor. The quality of an enzyme membrane greatly depends on the amount, ratio, and distribution control of E1 and E2 in it. Failure of the control results in poor linearity, sensitivity, and reproducibility (also known as interchangeability); the last one is extremely important in the manufacture of disposable biosensors. Many investigators tried to optimize the enzyme ratio in the membrane simply by adjusting the enzyme loadings (*1*−*7*), but the results were not always satisfying because the immobilization efficiency of the enzymes may differ from each other under the same immobilization conditions. On the basis of the studies of protein molecular structure, here we proposed a fusion enzyme method by gene manipulation to solve the problems.

Fusion protein technique has been widely used in the field of molecular biology and biotechnology for a variety of applications. Proteins or peptides which bind to specific molecular structures, such as glutathione S-transferase (*8*), maltose-binding protein (*9*), polyhistidine (*10*), strep-tag (*11*), are often employed as fusion partners to facilitate expression, purification, or immobilization of recombinant proteins. Green fluorescent protein (GFP) has been fused to some proteins as a reporter for protein

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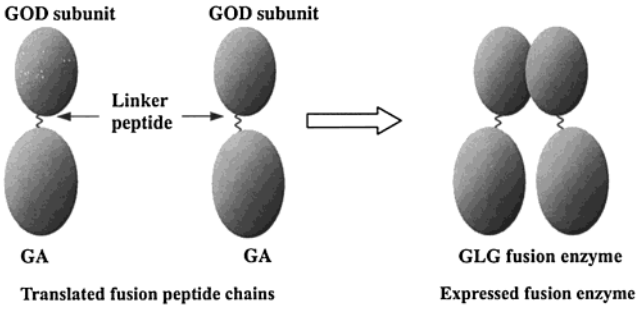

Figure 1. Schematic process of the fusion enzyme formation.

expression and localization (*12, 13*). Recently, there is a growing interest in a fusion enzyme possessing more than one catalytic function. Though the fragility and complexity of protein structures largely limit the success for accomplishing functionally active fusion enzymes, a few artificial fusion enzymes have been reported to exhibit noticeable performance in a concerned fashion (*14*−*19*). To our knowledge, there is no report concerning fusion enzyme application in biosensors yet.

The maltose biosensor, which employs GA and GOD as recognition elements, is one of the typical sequence biosensors (*20*). GA catalyzes hydrolysis of maltose to produce glucose that serves as specific substrate of GOD. Maltose is thus quantified by electrochemical measurement of oxygen consumption or release of hydrogen peroxide. GA is a monomer with a molecular weight of 99−112 kDa (*21*), and GOD is a dimeric flavoprotein with a molecular weight ranging from 150 to 186 kDa (*22*). The fusion enzyme constructed through gene fusion consists of two molecules of GA and one molecule of GOD. Since little information is available for predicting the structure and function change of a fusion enzyme, there is high risk in construction of such a complex hybrid protein. To avoid failure, a flexible linker peptide [-(Ser-Gly)$_5$-] was inserted between GA and GOD. This was thought to aid the independent folding of the two components (*23*). Figure 1 shows the schematic formation of GLG. Theoretically, GA and GOD would be adjacently immobilized with constant ratio by the proposed method if the fusion enzyme were successfully expressed. Reaction kinetics and reproducibility of the sequence biosensor should be accordingly improved. Details are reported herein.

MATERIALS AND METHODS

Materials. Restriction enzymes, DNA polymerase, and T4 DNA ligase were obtained from Takara and Promega. Lyticase, bovine serum albumin (BSA), O-dianisidine, soluble starch, PEG3350, glucose oxidase, and glucoamylase were purchased from Sigma. Yeast nitrogen base (YNB) was from Difco. All single strand oligonucleotides used in this study were obtained from Sangon (Shanghai, China). DNA purification columns and Q Sepharose Fast Flow ion exchange chromatography were from Qiagen and Pharmacia, respectively. A stirred cell system used for protein concentration was purchased from Filtron. *Aspergillus niger* 9029 containing GOD encoding region and plasmid pRD112 containing *Aspergillus niger* GA cDNA fragment were from American Type Culture Collection (ATCC). The methylotrophic yeast *Pichia pastoris* GS115 and its expression vector pPIC9 were kindly donated by professor Sheng-Li Yang. *Escherichia coli* DH5α was used for all bacterial transformations and plasmid propagations. All other reagents used were of analytical-reagent grade available.

Construction of Expression Vectors. All oligonucleotides were designed to incorporate proper restriction sites for cloning. Primers GODN-1 (5′-TACGTAAGCAATGGCATTGAAGCCAGC-3′) and GODC-1 (5′-AAGCTTCATGGAAGCATAATCTTCCAAG-3′), spanning the *Aspergillus niger* GOD encoding gene, were synthesized to amplify GOD gene with *Aspergillus niger* genome as template. Restriction sites SnaB I and Hind III were introduced to the N- and C-terminal primers, respectively. The fragment was first cloned into pGEM-T vector, and correct orientation was identified by restriction analysis. The resulting plasmid pGEM-TGOD was digested by SnaB I and Not I, and GOD encoding fragment was recovered and inserted into the SnaB I/Not I site of pPIC9, yielding GOD expression vector pPICGOD. The GA expression vector pPICGA1 was similarly constructed by using primers GAN-1 (5′TACGTAGCGACCTTGGATTCATGGTTGAG-3′) and GAC-1 (5′GCGGCCGCGAAATGGATTGATTGTCACC-3′) to amplify GA encoding sequence with plasmid pRD112 as template.

For the construction of GLG fusion gene, GOD gene was first amplified and cloned into pGEM-T vector as described above. The synthesized oligonucleotides containing [-(Ser-Gly)$_5$-] coding sequence (in bold) and an additional Hind III site (underlined) were annealed at 65 °C for 5 min to form a dsDNA fragment:

5′−AGCTC***AGCGGCTCTGGTTCCGGTAGCGGTTCCGGC**AAGCTTAGCGGTGC−3′

3′−G**TCGCCGAGACCAAGGCCATCGCCAAGGCCG**TTCGAATCGCCACGCCGG−5′

This resulting short fragment was inserted to replace the sequence between Hind III and Not I site in pGEM-TGOD, yielding plasmid pGEM-TGL. Using primers GAN-2 (5′-AAGCTTGCGACCTTGGATTCATGGTTGAG-3′) and GAC-1 (5′-GCGGCCGCGAAATGGATTGATTGTCACC-3′), the GA encoding sequence containing Hind III and Not I site at its N- and C-termini was amplified and cloned into pGEM-T vector. The GA fragment was obtained by digesting the resulting plasmid pGEM-TGA2 with Hind III and Not I and then ligated to pGEM-TGL linearized with the same restriction enzymes, yielding plasmid pGEM-TGLG. Because the Hind III restriction site immediately behind GOD gene had been silenced by a base mutation, which is indicated by an asterisk, in linker peptide coding sequence, the resulting plasmid pGEM-TGLG contains intact GLG encoding sequence. Plasmid pGEM-TGLG was digested with SnaB I and Not I, and the fragment encoding the fusion enzyme was cloned into the SnaB I/Not I site of pPIC9, yielding GLG fusion enzyme expression vector pPICGLG. The vectors, shown in Figure 2, contain expression cassettes of the 5′AOX1 promoter and 3′ AOX1 transcription termination fragment for regulated transcription and a-Factor signal peptide for secretion.

Preparation of *Aspergillus niger* Genomic DNA. *Aspergillus niger* genomic DNA was prepared by a modified method of Zhu et al. (*24*). *Aspergillus niger* cells were grown in YEP medium (peptone 20 g, yeast extract 10 g, glucose 20 g per liter) for 2 days. The mycelia harvested by centrifugation were washed with 10 mL of extracting solution (100 mM Tris·HCL, 40 mM EDTA, pH 8.5) twice and then mixed with 10 mL of extracting solution, 2 mL of 10% SDS, and 6 mL of benzyl chloride. The mixture was incubated at 50 °C for 1 h to digest the cell walls. The reaction mixture was supplemented with 6 mL of 3 M sodium acetate (pH 5.2) and kept in ice-bath for about 15 min. The mixture was then centrifuged at 10000 rpm for 15 min. After the mixture was deproteinated with PCA (phenol−chloroform−isoamyl alcohol,

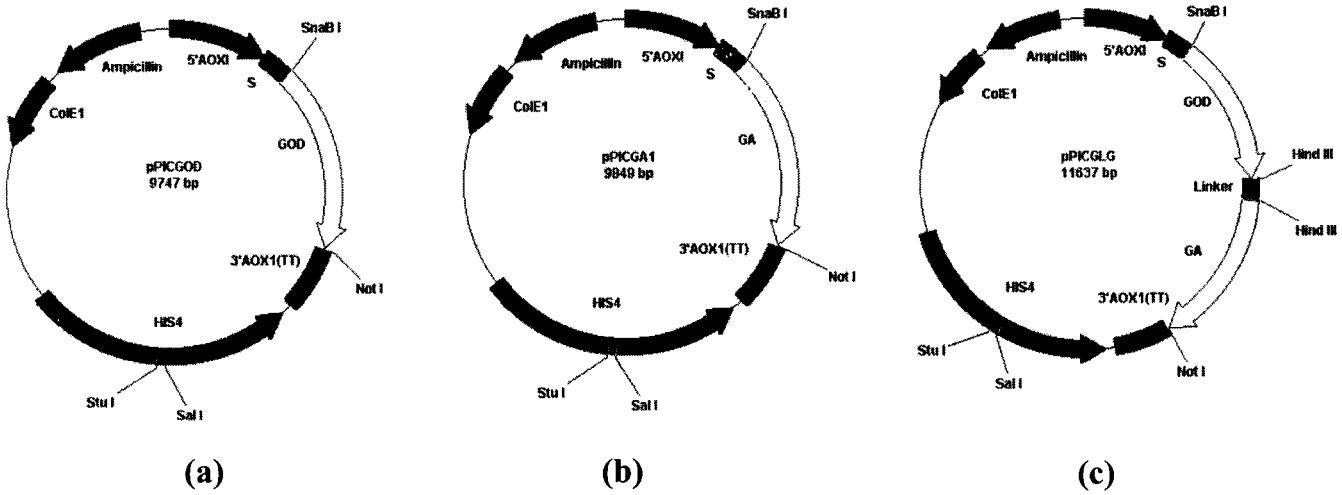

Figure 2. Constructed vectors for expression of (a) GOD, (b) GA, and (c) GLG fusion enzyme in *Pichia pastoris.*

25:24:1, vol/vol/vol), the DNA in the supernatant was precipitated with equal volume of 2-propanol.

Transformation of *Pichia pastoris.* pPICGOD, pPICGA1, and pPICGLG were digested with Stu I, and the linearized plasmid fragments containing GOD, GA, and GLG fusion gene, respectively, were introduced into GS115 as described previously (*25*). Integration of linearized pPICGOD, pPICGA1, and pPICGLG into the genome of GS115 was confirmed by PCR using yeast genomic DNA as templates.

Expression and Purification of the Yeast-Derived Enzymes. GOD, GA, and GLG were expressed respectively in *Pichia pastoris* under the control of AOX1 promoter. Yeast cells were cultured at 30 °C in 400 mL of MD medium (YNB 1.7 g, glucose 20 g, ammonium sulfate 5 g, and biotin 400 μg per liter) until the culture reached an optical density (λ = 600 nm) of 1.2−1.5. The cells were harvested by centrifugation and resuspended in 200 mL of MM medium (YNB 1.7 g, methanol 12 mL, ammonium sulfate 5 g, biotin 400 μg, and casamino acids 10 g per liter, pH 6.0) to induce the production of recombinant enzymes. After 5-day induction, the culture supernatant was concentrated to 30−40 mL using the FILTRON stirred cell system and dialyzed against 1000 mL of citrate buffer (pH6.0, 0.02 M) overnight.

The yeast-derived enzymes were purified using Q Sepharose Fast Flow ion exchanger chromatography. Initially, the column was washed with 20% (v/v) ethanol and then equilibrated with citrate buffer (pH 6.0, 0.02 M). The sample solution was applied to the column, and unbound protein was washed out with equilibration buffer. The protein bound to the column was eluted using a salt gradient in the equilibration buffer. Fractions containing enzyme activity were pooled and concentrated using a FILTRON stirred cell system. Homogeneity of the purified enzymes was checked by SDS−PAGE (10%), and a Bio-Rad protein assay kit was used to determine the protein concentration of each sample.

Enzyme Assays. For all enzyme assays one unit of activity was defined as the amount of enzyme that generated 1 μM of product per minute. GOD activity was determined using fresh *Aspergillus niger* GOD (Sigma) as standard. Assays were performed according to the method described by Frederick et al. (*26*). The steady-state kinetic parameters of purified GOD and GLG to glucose at pH 5.6, 30 °C were calculated by assaying initial velocities over a range of glucose concentrations from 10 mM to 100 mM under oxygen saturation condition. Lineweaver−Burk plots were used to determine the parameters on the assumption that simple Michaelis−Menter kinetics was followed.

GA was assayed by measuring the amount of glucose released from starch hydrolysis using 3,5-dinitrosalicilic acid (DNS) method (*27*). When assaying GA activity of the fusion enzyme, nitrogen bubbling was employed to remove oxygen from the reaction system, thereby blocking GOD activity. For determination of kinetic parameters, initial rates of starch hydrolysis catalyzed by yeast-derived GA and GLG were determined using up to 15 substrate concentrations from 0.2 mg/mL to 20 mg/mL.

Enzyme Immobilization. Immobilization of yeast-derived enzymes was carried out by a modified method of Fonseca (*28*). A glass slide with desired well assay was cleaned thoroughly and then coupled to γ-(aminopropyl)-triethoxysilane in a toluene solution. The glass slide was soaked in 100 mL of 2.5% (v/v) glutaraldehyde solution in phosphate buffer (pH 7.0, 0.05 M) for about 1 h at room temperature. The glutaraldehyde-activated slide was washed five times with phosphate buffer to remove unreacted glutaraldehyde. 5 μL of 50 μg/mL GLG fusion enzyme and a mixture of yeast-derived GOD and GA equivalent to corresponding enzyme activities of GLG (GA/GOD) were applied to the wells of the glass slide and incubated at room temperature for 4 h. Unbound enzymes were removed by washing thoroughly with phosphate buffer containing 0.05% Tween-20 and 0.5 M NaCl. Finally, 5 μL of substrate solution containing 4 mg/mL soluble starch, 0.01 mg/mL horseradish peroxidase, and 0.1 mg/mL *o*-dianisidine were added. Color change of the wells was observed and compared.

Preparation of the Enzyme Electrodes. The enzyme membranes were prepared by cross-linking method using glutaraldehyde as described in our previous report (*29*). Typically, 3 μL of 20% (w/v) BSA, 4 μL of 2.5% (v/v) glutaraldehyde solution, and 20 μL of enzyme solution were dropped onto a porous Teflon membrane, followed immediately by mixing and spreading on 7 mm diameter circular area with a tiny glass rod. Cross-linking reaction was allowed to perform for 1 h at room temperature. The enzyme membrane was then formed on the Teflon membrane. The enzyme constitutions in the electrodes were 18U of GOD for GOD electrode, 18U of GOD and 10U of GA for both GA/GOD electrode and GLG electrode.

The enzyme membrane was covered with a piece of nylon gauze and fixed on the tip of an oxygen electrode with an 'O' ring. The enzyme electrodes were soaked in

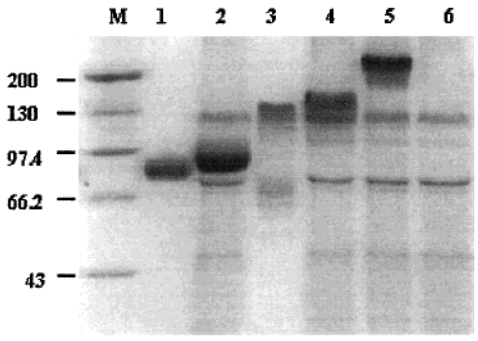

Figure 3. SDS−PAGE analysis of the total secreted proteins of *Pichia pastoris* cells. Lane M, high molecular weight protein marker (kDa); lane 1, *Aspergillus niger* GOD; lane 2, total secreted proteins of the yeast expressing GOD; lane 3, *Aspergillus niger* GA; lane 4, total secreted proteins of the yeast expressing GA; lane 5, total secreted proteins of the yeast expressing GLG; lane 6, total secreted proteins of the yeast carrying pPIC9.

citrate buffer (pH 4.8, 0.02 M), which was also used to prepare maltose solution, overnight to wash out unimmobilized enzymes and activated in 40 mM maltose solution at least 1 h before use.

Maltose Measurement. Maltose was measured using enzyme electrodes in combination with a flow injection analysis system (*30*). The working system was mainly composed of the enzyme electrode, amplified and digital display, peristaltic pump, injection unit, tubing system with citrate buffer (pH 4.8, 0.02 M) as carrying stream, and a printer. The carrier stream was pumped through the flow line at a flow rate of 2.0 mL/min, and 25 μL of sample solution was injected into the flow stream when the background current was stabilized. When the sample flowed through the surfaces of the electrodes, consumption of oxygen was measured through current drop.

RESULTS

Expression of GOD, GA, and GLG in *Pichia pastoris.* As a preliminary experiment, the expression vectors were transformed into host yeast separately. Positive transformants were confirmed by PCR analysis of the yeast genome. Expression of the recombinant proteins was induced by methanol. Enzyme assays indicated that pPICGOD, pPICGA1, and pPICGLG directed separately the synthesis and secretion of active GOD, GA, and GLG displaying both GOD and GA activity. The total proteins in the culture supernatant were analyzed by SDS−PAGE (Figure 3). It was found that GOD and GA secreted from *Pichia pastoris* migrated more slowly than the enzymes from *Aspergillus niger*. Yeast-derived GA and GOD monomer were visualized to have molecular weights of about 135 and 85 kDa, respectively. The difference of the molecular weight between proteins expressed in *Aspergillus niger* and *Pichia pastors* was also observed by other investigators (*26, 31*). They concluded that this was due to yeast-derived proteins tending to show substantially more N-linked carbohydrate than *Aspergillus niger* proteins. The protein band corresponding to GLG monomer on SDS−PAGE was observed at approximately 215 kDa. The molecular weight of GLG monomer agreed well with the sum of those of GA and GOD monomer. The native GLG which consists of two GA monomer and one GOD dimer has a molecular weight up to 430 kDa.

To optimize the production conditions, effects of pH and methanol concentration on the yield of GLG were studied. Although *Pichia pastoris* cells grew normally in all induction conditions tested, the optimal conditions for production of GLG were around 1.2% methanol added once a day and initial pH 5−6 (Figure 4). In addition, the activity of GLG did not increase appreciably upon induction for periods longer than 5 days in all shake flask studies. Hence induction in pH 6, MM medium for 5 days with daily addition of 1.2% methanol was chosen to produce GOD, GA, and GLG for further studies.

Purification of the Yeast-Derived Enzymes. After removal of the *Pichia pastoris* cells from the fermentation culture, the supernatant was concentrated by ultrafiltration and dialyzed to remove salt. GOD, GA, and GLG were purified using anion-exchange chromatography on a Q Sepharose Fast Flow column. Elution profile of all yeast-derived enzymes is shown in Figure 5a. It is clear that GOD, GA, and GLG require different elution time and ionic strength of the elution buffer. GOD was eluted from the column by using 0−0.1 M NaCl gradient solution, while GA and GLG were eluted by a NaCl gradient of 0−0.4 M. Among these enzymes, the fusion enzyme required the highest ionic strength. The purity of the proteins was checked by SDS−PAGE (Figure 5b).

Kinetic Properties of the GLG Fusion Enzyme. To examine the effect of protein fusion on the kinetic properties of both GOD and GA moieties of the fusion enzyme, the kinetic parameters of GLG were compared with those of individual enzymes from either *Pichia pastoris* or *Aspergillus niger*. Table 1 summarizes the kinetic constants of all enzymes studied here. GOD and GA secreted from *Pichia pastoris* had very high catalytic activity and showed about 1.6-fold and 2.0-fold increase of specific activity over that of the *Aspergillus niger* GOD and GA, respectively. Compared with the values of 426.62 U/mg protein and 238.34 U/mg protein measured for yeast-derived GOD and GA, the specific activities of the fusion enzyme toward glucose and soluble starch were determined to be 195.21 U and 116.56 U, respectively, per mg protein, showing that the catalytic activities of the fusion enzyme were successfully maintained. The k_{cat} and K_m of GLG to glucose were similar to the values determined for the yeast-derived GOD. The k_{cat} of GLG to soluble starch was 2.3 times as high as that of the yeast-derived GA due to the fact that 1 mol of GLG consists of 2 mol of GA. The higher turnover number of the fusion enzyme to soluble starch is thought to be advantageous for the sequential catalysis occurred in maltose biosensor. Compared with GOD and GA from *Aspergillus niger*, the yeast-derived GOD, GA, and GLG showed slightly lower affinity for substrates. However, the k_{cat} and k_{cat}/K_m determined for the yeast-derived enzymes were obviously higher than those of *Aspergillus niger* enzymes.

Enzyme Immobilization. Cross-linking and covalent bonding using glutaraldehyde are the most widely used enzyme immobilization methods. Immobilization of the yeast-derived enzymes was performed by depositing GLG and GA/GOD onto the glutaraldehyde-activated glass slide, respectively, with four duplicates. The immobilization profile would be a direct indication of whether the fusion enzyme had benefited the enzyme immobilization or not if so to what extent. After adding substrate solution containing soluble starch, horseradish peroxidase and *o*-dianisidine, the slide was incubated at 30 °C for more than 1 h to develop brown color, which in fact is a measure of the overall reaction efficiency catalyzed sequentially by GA and GOD. Relative recovery activities of GLG and GA/GOD were evaluated according to the darkness of the color spots. It was found that wells with immobilized GLG developed color much faster than those with immobilized GA/GOD. Visible color change appeared

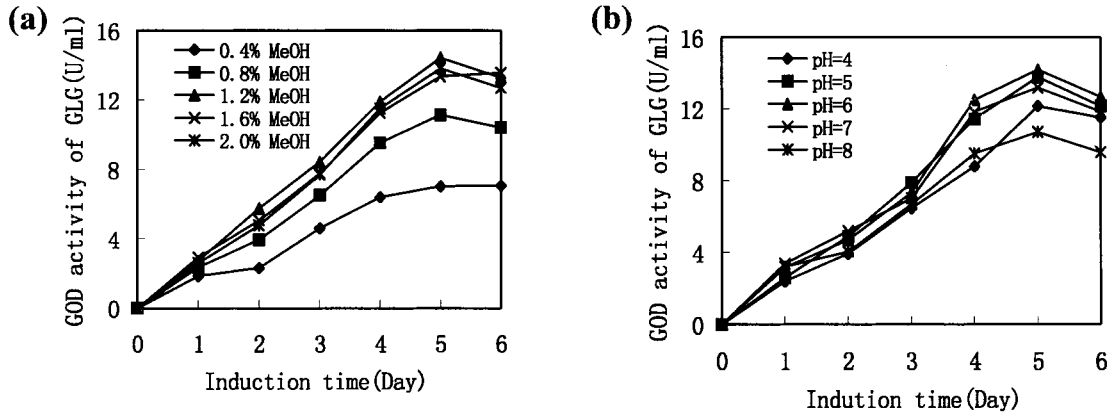

Figure 4. Effects of (a) methanol concentration and (b) pH on the production of GLG.

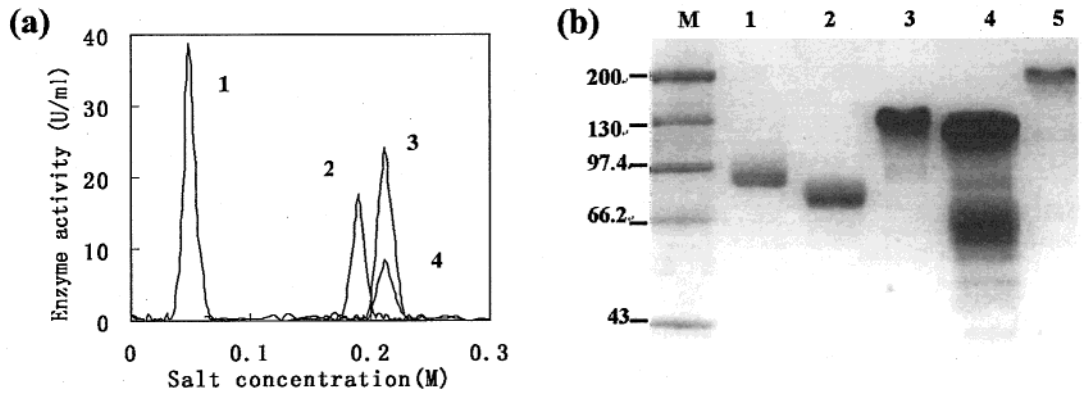

Figure 5. (a) Elution pattern of the yeast-derived enzymes. Peak 1 represents GOD; peak 2 represents GA; peak 3 and peak 4 represent GOD and GA activities of GLG, respectively. (b) SDS−PAGE analysis of the purified enzymes. Lane M, high molecular weight protein marker (kDa); lane 1, purified yeast GOD; lane 2, *Aspergillus niger* GOD; lane 3, purified yeast GA; lane 4, *Aspergillus niger* GA; lane 5, purified GLG.

Table 1. Kinetic Parameters of Native and Yeast-derived Enzymes

enzymes	specific activity (U/mg protein)	K_m (mM for GOD, mg/mL for GA)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1} for GOD, s^{-1} mL.mg^{-1} for GA)
A. niger GOD	266.31	33.44	2303.56	68.89
P. pastoris GOD	426.62	38.25	3492.66	91.31
A. niger GA	118.75	1.347	557.33	413.76
P. pastoris GA	238.34	1.703	1123.71	659.84
GOD of GLG	195.21	37.16	3314.13	89.19
GA of GLG	116.56	1.774	2601.33[a]	1466.36

[a] The k_{cat} value was calculated per GLG, which contains two GA active sites.

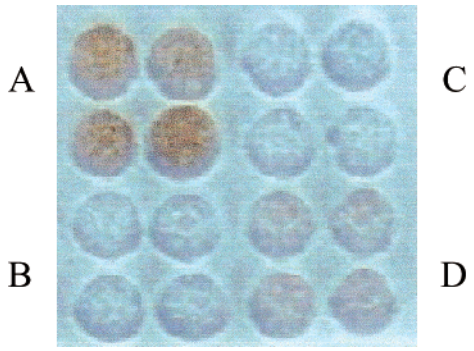

Figure 6. Immobilization of the enzymes on a glass chip. The spots that developed brown show positive reactions, others show negative reactions, and each loaded four duplicates. Experimental detail is in the text. (A) denotes the detection of GLG; (B) denotes the detection of GOD; (C) denotes the detection of GA; (D) denotes the detection of the mixture of GA and GOD (GA/GOD).

within 1 h for the fusion enzyme, but only a faint color developed in the spots with immobilized GA/GOD even after 5 h incubation (Figure 6). The result demonstrates that GLG fusion enzyme is obviously superior to GA/GOD in terms of immobilization efficiency. No detectable signal was observed in the spots of negative controls where the yeast-derived GA and GOD were immobilized individually on the glass chip with the same procedure.

Evaluation of the Enzyme Electrodes by Measuring Maltose. The responses of the three kinds of enzyme electrodes to maltose solution were investigated. As shown in Figure 7, the GOD electrode did not response to maltose, while the response values of GLG electrode and GA/GOD electrode were proportional to maltose concentration. To the same maltose solution, the response signal of GLG electrode is higher than that of GA/GOD electrode. A linear response range up to 40 mM was obtained for GLG electrode and 30 mM for GA/GOD electrode. The increased signal level and extended measuring linearity of the GLG system should be attributed to the higher immobilization efficiency of GA and GOD as well as their proximity effect in the enzyme membrane.

To investigate the precision and reproducibility of the electrodes, both GLG electrode and GA/GOD electrode were fabricated with six repeats, and each electrode was used to measure 10 mM maltose solution five times in succession. Good precision was obtained for both GLG electrode and GA/GOD electrode. The variation coefficients of the response values varied from 0.6% to 4.2%

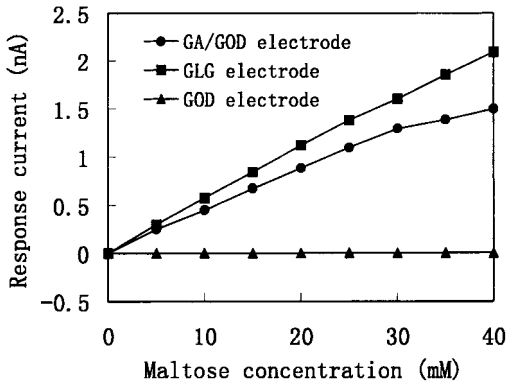

Figure 7. Responses of the enzyme electrodes to maltose.

Table 2. Analysis of Reproducibility of Enzyme Membranes

electrode	the responses of six enzyme membrane[a] (nA)	variation coeff of the enzyme membranes (%)
GA/GOD	0.346, 0.489, 0.401, 0.395, 0.547, 0.528	18.1
GLG	0.527, 0.613, 0.583, 0.645, 0.505, 0.577	9.1

[a] Each response value here was the mean of five repeated measurements to 10 mM maltose.

for six GLG electrodes and from 1.3% to 5.4% for six GA/GOD electrodes. However, the reproducibility, which represents the interchangeability between enzyme membranes, of GLG membranes was much better than that of GA/GOD membranes. Compared with the values of 18.1% calculated for GA/GOD membranes, the variation coefficient of GLG membranes was determined to be 9.1% (Table 2).

DISCUSSION

The large hybrid fusion enzyme GLG was successfully constructed in this study. The linker peptide composed of serine and glycine was designed to minimize the steric hindrance between GA and GOD so as to provide enough space for both proteins to remain in native conformation. This was indirectly proved by comparison of its catalytic activities with the native GA and GOD expressed in the same host. The same linker had been successfully used in our previous work where the alkaline phosphate was actively fused to strep-tag through it (*32*). Our observations suggest that the linker peptide play an important role in the successful expression of the fusion enzyme. The resulting fusion enzyme GLG maintained typical characteristics of both GA and GOD. No any detectable activity of the fusion enzyme was obtained at cellular level without the linker insertion (data not shown). On the other hand, it is interesting that this heterogeneous hybrid protein up to 430 kDa can still correctly fold in yeast cell and secrete. The mechanism is worth studying.

For process development, GOD and GA have also been separately expressed in the same host. It is presumed that all yeast-derived enzymes here were hyperglycosylated according to their lower electrophoretic mobility (Figures 3, 5b). Yeast is known to hyperglycosylate some secreted foreign protein, especially those using α-Factor signal leader for secretion (*26, 31*). However, at present the reason for the differential glycosylation of protein between yeast and *Aspergillus niger* is still not clear. A detailed kinetic comparison of the various enzymes demonstrated that the yeast-derived GOD and GA showed increased specific activity and catalytic efficiency than the *Aspergillus niger* enzymes.

In the fabrication of enzyme sensors, the immobilization of enzymes on the electrode surface is a crucially important step, which determines the performance characteristics of the biosensors. Sequence biosensors require coimmobilization of more than one enzyme in the same membrane. The discrepancy of immobilization efficiency between enzymes, without doubt, will affect the performance of the biosensor, especially in case one enzyme having relatively poor immobilization efficiency. The fusion enzyme offers several advantages over individually coimmobilized enzymes. First, it provides fixed molecular ratio of immobilized enzymes, ensuring the desired activity of the enzyme membrane. The compositions and structures of both *Aspergillus niger* GA and GOD were resolved and released (*26, 33*−*36*). There are 13 and 30 lysine residues in the structures of GA and GOD, respectively. Most of them are located on the surface of the structures (Figure 8). Lysine provides an amino group for coupling to glutaraldehyde, a bifunctional reagent for cross-linking reaction or covalent bonding. It clearly demonstrates why GA is more difficult to be immobilized than GOD using glutaraldehyde. In case of GLG, neither GA nor GOD could escape whichever reacted with the bifunctional reagent during immobilization process. This assumption has been proved by the evidence shown in Figure 6, where the recovery activity of the sequence reaction catalyzed by GLG was much higher than that of GA/GOD, despite of the same enzyme activity loading in both cases. Second, the ratio control of GA and GOD activity greatly improved the homogeneity of the enzyme membranes from batch to batch of manufacture (Table 2), which is one of the main factors affecting the quality of enzyme sensor. Poor homogeneity results in difficulty of interchangeability between the enzyme membranes. It is, as mentioned above, the major premise to develop

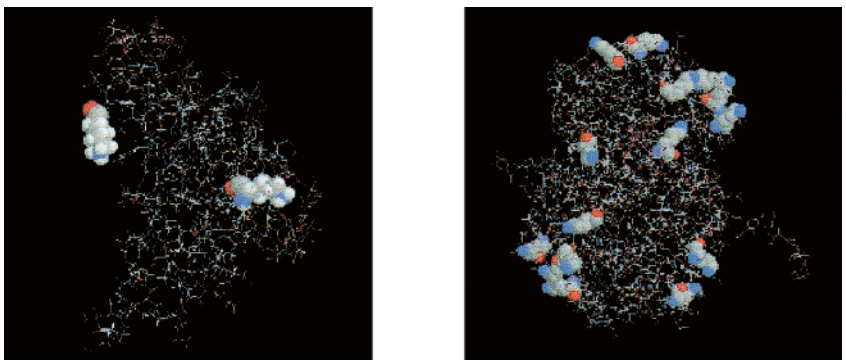

Figure 8. The crystal structures of (a) granular starch binding domain of *Aspergillus niger* GA bound to *β*-cyclodextrin (Sorimachi et al.) and (b) *Aspergillus niger* GOD subunit (Hecht et al.). Lysines are shown in space-filling models.

the disposable biosensors where no calibration step can be carried out when in use. Similar requirement can be met in construction of other biomolecular devices, such as protein chips. Third, the distance between the active centers of the fused enzymes is shortened and controlled, which makes the intermediate produced by the first enzyme readily available to the second enzyme, leading to a faster reaction rate and thus higher response signal or sensitivity of the biosensor (Figure 7). It is argued that fusion enzymes may not function at their highest active level because the optimal working conditions for the enzymes to be fused may differ from each other. The working condition, however, could be compromised if there is no better choice.

Our findings suggest that GA and GOD can be effectively used as fusion partners to construct a genetically artificial fusion enzyme despite their complex tertiary structure. And, in conclusion, the method proposed in this study offers a basis to develop high quality sequence biosensors as well as other sequential enzyme reaction applications.

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