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An Improved Method for Production of Recombinant Human Glutamic Acid Decarboxylase 65 for Use in Phytopharmaceutical Assessment*

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Abstract

The pharmacological activity of neuroactive phytochemicals on recombinant human glutamic acid decarboxylase 65 (hGAD65) was investigated. GAD catalyzes the conversion of glutamic acid to γ-aminobutyric acid (GABA), which acts as an important inhibitory neurotransmitter in the central nervous system (CNS). We describe an improved method in which recombinant hGAD65 was expressed at high levels using a maltose binding protein (MBP) fusion system. The expression and purification process was superior to the commonly used glutathione*S*-transferase (GST) fusion partner. The *in vitro* system developed here detected both enzyme inhibition and stimulation, under varying pyridoxal-5′-phosphate (PLP) concentrations. The known GAD inhibitor, 3-mercaptopropionic acid, was tested as a positive control and had an $IC_{50} = 12.3$ μM. Phytochemicals were tested (10 μg/mL) for their effects on *in vitro* hGAD65 activity. Minor inhibition was seen with the ethanol extract of *Panax quinquefolius* L. (ginsenosides) (23%), betulinic acid (27%), and valerenic acid (20%). An increase in hGAD65 activity by approximately 20% was observed with bilobalide and asiaticoside. As a result, these small changes in GAD activity may have physiologic implications. The possibility that phytochemicals influence GABAergic neurotransmission *in vivo* and the mechanisms by which it may occur is discussed.

Keywords: Asiaticoside, bilobalide, ginsenosides, maltose binding protein fusion, recombinant human GAD65, valerenic acid.

Introduction

Glutamic acid decarboxylase (GAD; EC 4.1.1.15) is a pyridoxal-5′-L-phosphate (PLP)-dependent enzyme responsible for the synthesis of γ-aminobutyric acid (GABA), a crucial inhibitory neurotransmitter in the vertebrate brain. GAD produces GABA from the decarboxylation of glutamic acid (Erlander & Tobin, 1991; Martin & Rimvall, 1993). Disturbances in GABA levels are responsible for a host of neurologic diseases (Nakanishi, 1992; During et al., 1995; Luddens et al., 1995; Wong et al., 2003). In addition, GABA is involved in development and regulation of neuroendocrine function (Trudeau et al., 2000; Tobet et al., 2001).

In the mammalian nervous system, two isoforms of GAD, GAD67 and GAD65, are present (Erlander et al., 1991). The 65-kDa isoform (GAD65) has been implicated in epilepsy (Kash et al., 1997), and in fear and anxiety (Stork et al., 2000, 2003) in mice. GAD65 is also important from a different perspective as it is a major autoantigen in type 1 diabetes (Baekkeskov et al., 1990; Kaufman et al., 1992). Autoantibodies to the islet-cell GAD65 are detected in insulin-dependent diabetes mellitus patients several years before the onset of clinical symptoms of the disease (Baekkeskov et al., 1990). Moreover, the presence of GAD autoantibodies is also associated with a rare neurologic disorder called stiff-person syndrome (Solimena et al., 1990).

Current expression methods for recombinant human GAD65 have used the glutathione *S*-transferase (GST) fusion protein system. However, overall yield is relatively low, and large-scale purification protocols using 100 L bacterial

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cultures are required (Davis et al., 2000). Further, GAD65 is a membrane-bound protein, making it difficult to purify via conventional methods. The purification process has been enhanced by incubation of transformed bacteria at a reduced temperature and by addition of sorbitol and betaine to the culture broth; all of which have prevented the formation of inclusion bodies (Buss et al., 2001). These modifications can slow down the overall procedure. The growth time for the bacteria cells is doubled, and the resultant soluble proteins are dissolved in a viscous solution that also runs slowly on the protein purification column. We recently reported the expression of high levels of active goldfish GAD65 using the maltose binding protein (MBP) fusion system (Sardana et al., 2006). In the construction of fusion protein discussed here, MBP was selected because it is known to promote the solubility of some polypeptides to which it is fused (Kapust & Waugh, 1999). Given that human GAD65 is a major target in neurologic disorders such as anxiety and epilepsy, production of sufficient quantities of active enzyme is required for pharmacological assessment.

Based on the current hypothesis of GABAergic neurotransmission, CNS-related disorders may be controlled by altering central GABA levels. We recently reported activity of several anxiolytic botanical extracts, including American ginseng (*Panax quinquefolius*), valerian (*Valeriana officinalis*) and gotukola (*Centella asiatica*) on in vitro GAD activity using rat brain homogenates (Awad et al., 2007). Thus, it was of interest to our group to investigate the effects of isolated phytochemicals from these active botanicals on recombinant human GAD activity. In the field of phytopharmacology, it is increasingly critical to find new and improved techniques to evaluate the biological activity of natural-based products and compounds. The system we describe in this paper has such applications.

The objectives of our study were to (1) construct a new expression vector for the complete human GAD65 (hGAD65) using pMAL-c2X in *Escherichia coli* (*E. coli*); (2) produce higher quantities of purified active recombinant human GAD65 using the MBP expression system for *in vitro* analyses; and (3) determine the pharmacological activity of selected phytochemicals with known neurologic function to better define their safety and provide indication of their molecular mechanism of action.

Materials and Methods

Construction of the hGAD65 expression vector using pMAL-c2X

A recombinant pET-5C plasmid containing the hGAD65 insert was obtained from A.J. Tobin (see Bu et al., 1992). The 1757-bp hGAD65 insert from the pET-5C plasmid was subcloned into the pMAL-c2X protein fusion and purification system (New England Biolabs, Ipswich, MA, USA) for cytoplasmic targeting in *E. coli*. The hGAD65 insert was removed from the pET-5C vector by restriction digest with *BamH* I (Invitrogen, Burlington, Canada) and separated via gel electrophoresis. The hGAD65 band was excised from the gel and purified using QIAquick gel extraction kit (Qiagen, Mississauga, Canada). Digestion with *BamH* I generated 5′-overhangs that were filled in to generate a blunt-end fragment using the Large Fragment (Klenow) of DNA polymerase I (Invitrogen). The hGAD65 insert was then purified via phenol-chloroform extraction and ligated with *Xba* I phosphorylated linkers (New England Biolabs). The linker concatemers were digested using *Xba* I (Invitrogen), and the resulting hGAD65 insert flanked by monomeric *Xba* I linkers was gel purified, as described above. The pMAL-c2X vector was prepared by cleavage with *Xba* I and dephosphorylation with calf intestinal alkaline phosphatase (Invitrogen) followed by phenol-chloroform extraction. The hGAD65 insert with *Xba* I linkers was cloned into the linearized pMAL-c2X vector in an overnight ligation reaction at room temperature. The ligation reaction was then used to transform subcloning efficiency DH5α chemically competent *E. coli* (Invitrogen). Individual bacterial colonies were tested for the presence of the hGAD65 insert via colony PCR with sequence-specific primers (forward 5′-CTCTGGCTTTTGGTCTTTCG-3′, reverse 5′-TGACATCCACTTTGGAGCAG-3′). Colony PCR was again used to test the hGAD65 positive colonies for the correct orientation of insertion using a forward primer specific to the mal E site on the pMAL-c2X (5′-GGTCGTCAGACTGTGATGAAGCC-3′) vector and the hGAD65-specific reverse primer. Positive colonies were then picked and grown in liquid cultures overnight. The DNA was isolated and sequenced at the junction to confirm that the hGAD65 insert was in-frame with the maltose binding protein. The subcloning from the original pET-5C vector and the use of *Xba* I linkers resulted in a total of 11 amino acids between the factor Xa cleavage site and the start codon of the hGAD65 sequence.

Expression and purification of recombinant fusion proteins

The expression and purification of recombinant hGAD65 was compared using the pMAL-c2X and the pGEX-3X plasmids, containing the MBP and GST fusion partners, respectively. The optimized conditions for each system have been previously published (Buss et al., 2001; Sardana et al., 2006). To accurately compare the total protein yield and the relative enzymatic activity of each recombinant hGAD65, the two optimized methods were conducted simultaneously. Chemically competent DH5α *E. coli* (Invitrogen) cells were also used for both systems.

MBP-hGAD65 system

Single bacterial colonies transformed with the pMAL-c2X–hGAD65 vector were grown overnight in 10 mL Lennox

broth (LB) (Invitrogen) supplemented with 2 g/L glucose and 100 μg/mL ampicillin. The following day, 99 mL of media (LB/glucose, 100 μg/mL ampicillin) was inoculated with 1 mL of the overnight culture ($OD_{600} = 0.1$). The culture was incubated at 37°C with shaking until the OD_{600} reached ~0.5 (approx. 1.43×10^7 cells), at which time 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added. The bacterial cells were then grown at 20°C overnight and recovered by centrifugation (5000 rpm, 45 min, 4°C) the next morning. Cell pellets were resuspended in 10 mL buffer [20 mM Tris-HCl, 200 mM NaCl, and 1 mM ethylenediaminetetraacetic acid (EDTA)] . The samples were frozen, stored overnight at –20°C, and thawed the next day on ice. To minimize proteolysis, the established procedures for protein isolation were followed as instructed in the technical manual from New England Biolabs. Thus, EDTA was included in the lysis buffer to inhibit calcium-dependent proteases. Proteolysis was further limited by culturing bacteria at the lower temperature of 20°C. Samples were sonicated with 10–15 short pulses of 15 s or less. To collect the soluble proteins, samples were centrifuged (11,000 $\times$ g, 30 min, 4°C) and the supernatants collected.

To purify the fusion proteins, a column containing 3 mL amylose resin (New England BioLabs) was prepared. The column was washed at room temperature with 120 mL buffer (20 mM Tris-HCl, 200 mM NaCl, and 1 mM EDTA) and then moved to 4°C. The supernatant containing the soluble proteins was loaded twice onto the column. The column was washed 12 times and subsequently moved to room temperature. The fusion protein was eluted with the column buffer, with the addition of 10 mM maltose. A total of eight fractions of 2 mL each were collected, and aliquots of 100 μL were used for protein quantification and analysis on SDS-PAGE gels.

GST-hGAD65 system

Expression and purification of hGAD65 was performed as described earlier (Buss et al., 2001). This is a well-characterized recombinant human GAD (Bu et al., 1992; Qu et al., 1998; Davis et al., 2000). Single bacterial colonies containing the pGEX-3X–hGAD65 vectors (kind gift of Drs. N. Tillikaratne and A.Tobin, Brain Research Institute, UCLA) (Bu et al., 1992) were grown overnight in 10 mL LB (Invitrogen) containing 1 M sorbitol, 1.7 mM betaine and 100 μg/mL ampicillin. The following day, 99 mL media (LB/sorbitol/betaine supplemented with 100 μg/mL ampicillin) was inoculated with 1 mL of the overnight culture ($OD_{600} = 0.1$). The culture was incubated at 37°C with shaking until the OD_{600} reached ~0.3 (approx. 0.96×10^7 cells), at which time 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added. The bacterial cells were then grown at 20°C overnight and recovered by centrifugation (5000 rpm, 45 min, 4°C) the next day. Cell pellets were resuspended in 10 mL buffer [PBS, 1% Triton X-100, and 10 mM 2-(2-aminoethyl) isothiouronium bromide (AET), a protease inhibitor]. Samples were sonicated with 10–15 short pulses of 15 s or less. To collect the soluble proteins, samples were centrifuged (11,000$\times g$, 30 min; 4°C) and the supernatant collected.

To purify the fusion proteins, a column containing 3 mL glutathione sepharose 4B resin [Amersham Biosciences (GE Healthcare), Baie d'Urfe, QC, Canada] was prepared. The column was washed at room temperature with 120 mL of a buffer (PBS, 1% Triton X-100, and 10 mM AET) and then moved to 4°C. The supernatant containing the soluble proteins was loaded twice onto the column. The column was washed 12 times as above and subsequently moved to room temperature. The fusion protein was eluted with 50 mM Tris-HCl (pH 8.0) and 5 mM reduced glutathione. A total of eight fractions of 2 mL each were collected, and aliquots of 100 μL were used for protein quantification and analysis on SDS-PAGE gels.

SDS-PAGE analysis and silver staining

Fractions were collected, verified by SDS-PAGE (Laemmli, 1970; Sambrook et al., 1989), and stored at –80°C until further analysis. Protein bands were detected using the Bio-Rad silver stain kit [Bio-Rad Laboratories (Canada) Ltd., Mississauga, Canada]. The protein ladder was a low-range silver stain SDS-PAGE standard from Bio-Rad.

Comparison of protein yield and activity

Protein quantification

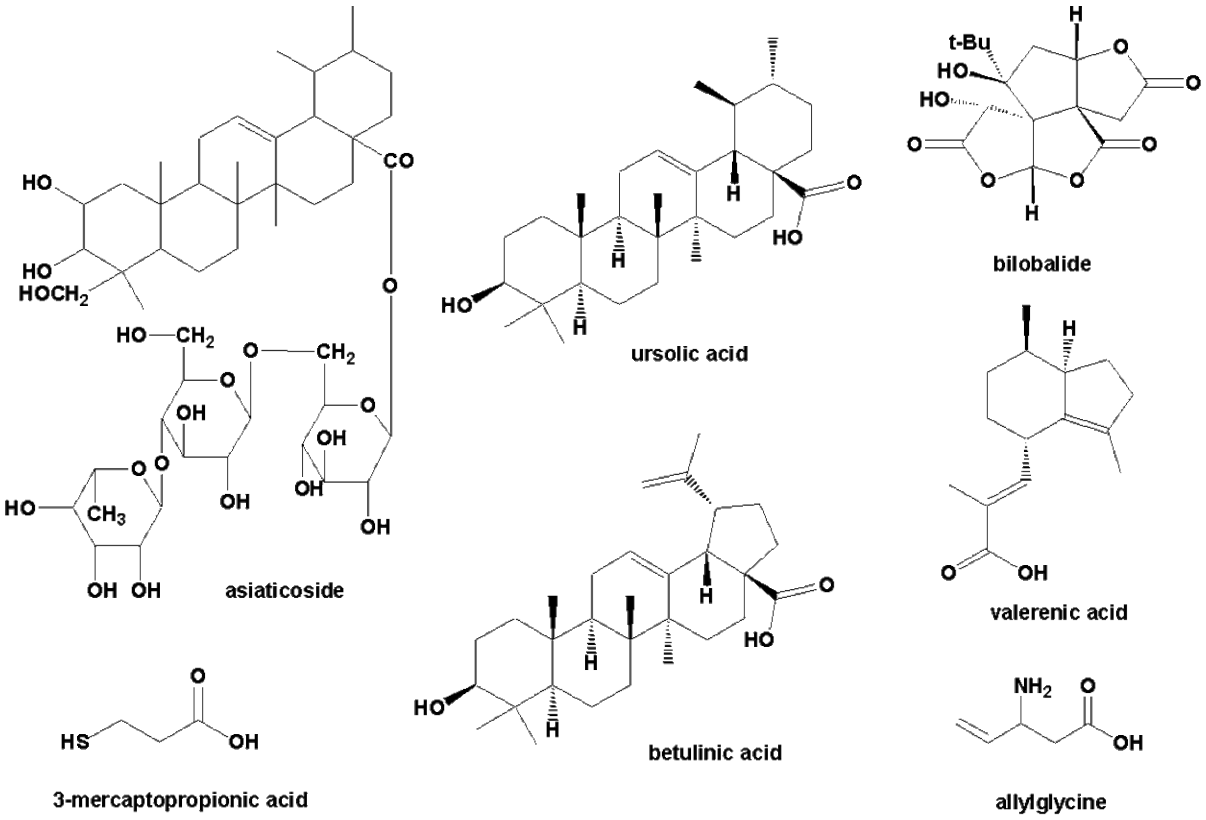
Total protein from each fraction collected was analyzed using the Bio-Rad protein assay kit, which is based on the method of Bradford (1976). Absorbance was measured at 595 nm using a SpectraMax M5 spectrophotometer, with SoftMax Pro Software version 4.8 (Molecular Devices Corporation, Sunnyvale, CA. USA). An eight-point standard curve (0–1 mg/mL) was made using the reference reagent and used to determine the protein concentration (μg/mL) in each sample.

In vitro recombinant hGAD65 activity

Specific enzyme activity of each fraction was assessed using the *in vitro* radiometric method (Sardana et al., 2006). Recombinant MBP-hGAD65 and GST-hGAD65 fractions were cleaved with factor Xa (New England Biolabs, Beverly, MA, USA) for 4 h at 4°C and used for each reaction. To compare both fusion systems, 60 μM PLP was used, an amount in excess of that required for optimum activity (Buss et al., 2001), ensuring that PLP concentration was not a limiting factor.

Assessing the effects of neuroactive phytochemicals on hGAD65 activity

Selected phytochemicals with potential neurologic action were tested to determine their effect on *in vitro* hGAD65



Ginsenoside	R_1	R_2	R_3
Rb_1	-Oglc(2-1)glc	-H	-Oglc(6-1)glc
Rb_2	-Oglc(2-1)glc	-H	-Oglc(6-1)arap
Rc	-Oglc(2-1)glc	-H	-Oglc(6-1)araf
Rd	-Oglc(2-1)glc	-H	-Oglc
Re	-OH	-Oglc(2-1)rha	-Oglc
Rg_3	-Oglc(2-1)glc	-H	-OH

Figure 1. Chemical structures of compounds tested in the hGAD65 *in vitro* assay. Glc; β-D-glucose; Rha, α-L-rhamnose; Arap, α-L-arabinose(pyranose); Araf, α-L-arabinose (furanose).

activity (Fig. 1). Valerenic acid and asiaticoside (Indofine Chemical Company Inc., Somerville, NJ, USA) are biologically active secondary metabolites from *Valeriana officinalis* and *Centella asiatica*, respectively (Houghton, 1999; Wijeweera et al., 2006). Both botanical extracts showed stimulatory activity on total rat brain GAD *in vitro* (Awad et al., 2007). Thus, these two marker phytochemicals were tested to determine if they had the same stimulatory effect on human GAD65.

Each standard was dissolved in 99% ethanol, and serial dilutions were prepared. An aliquot of 2.3 μL of each dilution was added to the reaction vial (final concentrations: 0, 0.1, 0.3, 1, 3, 10, and 30 μg/mL). This volume ensured that the final ethanol concentration was less than 1% in each vial. The *in vitro* GAD assay was followed as described above. However, before the addition of radioactive glutamic acid, the vials were initially incubated with the pure compound and 15 μM PLP for 15 min at 37°C to allow for potential interaction with the enzyme. Our previous work (Sardana et al., 2006) demonstrated peak activity of hGAD65 at approximately this dose of PLP. Asiaticoside did not inhibit GAD activity even at the highest dose of 30 μg/mL. Therefore, a second experiment was done with a single dose of asiaticoside (10 μg/mL) under various PLP concentrations (0, 3.75, 7.5, and 15 μM) to determine if stimulation could be detected at lower PLP levels.

In addition, the following materials of interest were tested (final concentration, 10 μg/mL) as they have also shown neurologic actions. A standardized ethanol extract from the roots of North American ginseng, *Panax quinquefolius* L. (Brody Farm, ON, Canada; batch no. M-EG-12–2004) containing approximately 40% ginsenosides: Rb_1 (59.1%), Rb_2 (2.0%), Rc (11.4%), Rd (6.9%), Re (18.6%), and Rg_3 (2.0%); bilobalide, a sesquiterpene found in *Ginkgo biloba*; and ursolic acid and betulinic acid (Sigma Chemical Company, St. Louis, MO, USA), two naturally occurring triterpenes found in a variety of plant species such as *Melissa officinalis* and *Souroubea sympetala*. Known GAD inhibitors, 3-mercaptopropionic acid (3-MPA) (Wu, 1976) and allylglycine (Wang et al., 2007), were employed as positive controls for enzyme inhibition. Each was dissolved in water and tested in triplicate at 0.1, 1, 10, and 100 μM.

Statistical analysis

One-way analysis of variance (ANOVA) using S-PLUS 7 software (Insightful Corporation, Seattle, WA, USA) was used to determine statistical significance for the asiaticoside-PLP experiment, using a pairwise comparison and Bonferroni adjustment. Linear regression using Probit software (Hubert & Carter, 1990) was performed to determine the IC_{50}value of 3-MPA ($\pm$ 95% confidence interval; CI).

Results

Construct design, expression and purification of hGAD65

The coding portion of the human cDNA was cloned in the pMAL-c2X vector downstream from the MBP sequence. This resulted in fusion of a MBP sequence with that of the hGAD65 sequence. The pMAL-c2x/hGAD65 construct (Fig. 2) was made to target the fusion protein to the cytoplasm. *E. coli* bacteria were cultured, and growth was monitored by measuring the absorbance at OD_{600} (Fig. 3). After inducing the expression of fused coding sequences in the bacteria, the soluble proteins were extracted and were passed over an affinity column with amylose resin to purify the fusion protein. A total of eight 2-mL fractions were collected. Aliquots from these fractions were analyzed on a 10% SDS gel (Fig. 4, shows fractions 1–4) and visualized with silver staining. Fractions 1–7 contained the predicted fusion protein band of ~107 kDa (65 kDa from hGAD65 + 42 kDa from MBP) in varying amounts. This fusion protein band migrates just above the 97-kDa-size molecular weight marker. In addition, a protein band appeared around 42 kDa, which is generally obtained with the MBP fusion system (see manufacturer's protocol) and is the MBP that gets cleaved from the fusion partner by the inherent action of a protease(s) in the bacterial extract. A part of it could also originate from the

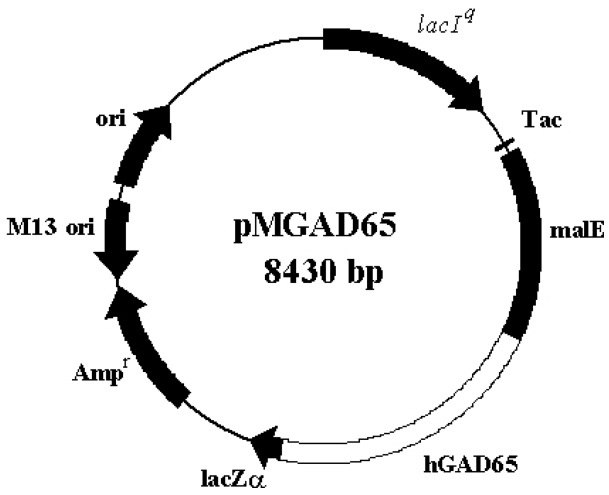


Figure 2. The pMAL-c2x/hGAD65 fusion construct. The hGAD65 coding sequence was fused in-frame with the DNA sequence for maltose binding protein, malE, under the control of "Tac" promoter.

wild-type MBP produced in bacteria. Running an additional gel with a commercial aliquot of maltose binding protein further substantiated this. Additionally, there were almost no other protein bands seen in these samples. It is thus concluded that the fractions collected were significantly pure.

The pGEX-3X/hGAD65 construct, on the other hand, coded for fusion of hGAD65 with a GST sequence. This fusion was expressed and purified according to an earlier publication (Buss et al., 2001). From this protocol, fractions 2–8 contained the predicted fusion band of ~ 92 kDa (65 kDa from hGAD65 + 27 kDa from GST). However, as shown in Figure 4 (fractions 2–5), there are a large number of contaminating bands of various sizes present throughout

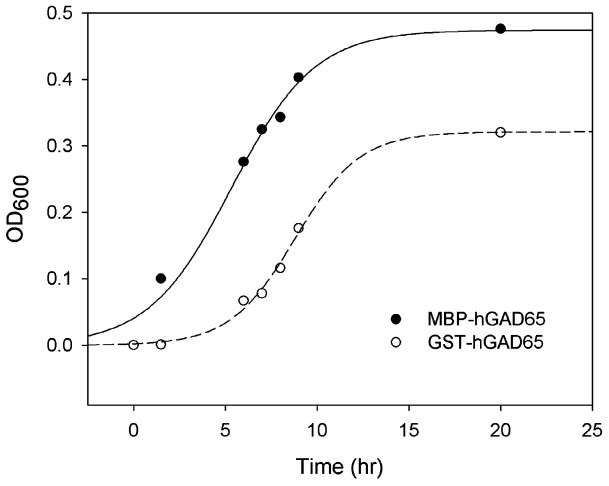


Figure 3. Growth curves of *E. coli* containing either MBP-hGAD65 or GST-hGAD65 fusion proteins, expressed as the absorbance at 600 nm.

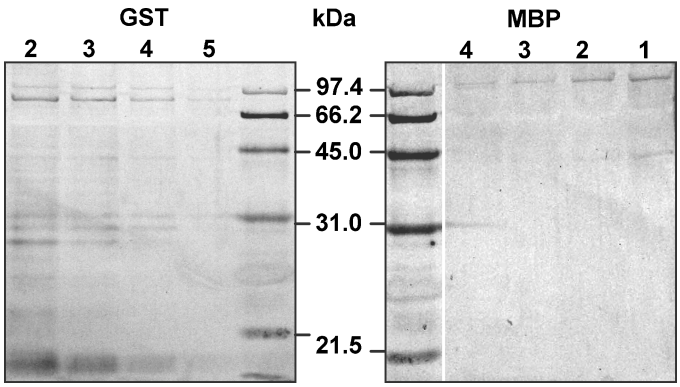


Figure 4. SDS-PAGE gels stained with silver reagent to detect GST-hGAD65 (fractions 2–5) and MBP-hGAD65 (fractions 1–4) fusion proteins collected after purification on glutathione sepharose and amylose columns, respectively. Molecular mass markers are outlined in the center. The GST-hGAD65 fusion appears as a dark band below the 97.4 kDa, and the MBP-hGAD65 fusion migrates just above it. The GST and MBP fractions were run on two different gels. They were handled similarly and stained on the same day. Note the presence of a large number of contaminating bands in the GST fractions.

the gel. For this reason, it is concluded that the GST fractions collected are less pure than those collected from the MBP system.

Comparison of hGAD65 activity: MBP versus GST

The *in vitro* GAD assay determined the total specific activity for the MBP and GST fractions to be 0.38 and 0.24 nmol min^{-1} mg $protein^{-1}$, respectively. This represents a 1.6-fold increase in hGAD65 activity purified via the MBP construct. Thus, it is probable that the production of active hGAD65 using the MBP system would increase proportionally if performed at a larger scale. Importantly, the rate of hGAD65 activity per amount of total protein was comparable between the two expression systems. Therefore, overall enzyme activity is not affected by the fusion protein construct used. However, the total yield and purity of hGAD65 in the MBP fractions is superior. Further, bacterial cells grown under the MBP system grew twice as fast as those containing the GST fusion. This reduced culture time facilitates protein purification and makes working with the MBP system easier and more favorable. Based on these results, we believe this improved method is of considerable value for *in vitro* pharmacological evaluation.

Pharmacological assessment of phytochemicals using hGAD65

Recombinant hGAD65, purified using the MBP fusion system, was chosen to assess the direct effects of selected phytochemicals on enzyme activity. The known GAD inhibitor, 3-MPA, was used as positive control for inhibition and had an $IC_{50} = 12.3$ μM (95% CI = 9.6, 16.2) (Fig. 5). Allylglycine was also tested at a range of concentrations, however, no inhibition was observed even at the highest dose of 100 μM. The marker phytochemicals affected hGAD65 activity to varying degrees (Table 1). Betulinic acid had the greatest inhibition (27%), although the standard deviation was fairly high. This was followed by the ginsenosides (23%), valerenic acid (20%), and ursolic acid (11%). Interestingly, pre-treatment with bilobalide resulted in an increase in activity by approximately 18%. Additionally, when valerenic acid was tested at varying concentrations, it did not exhibit a dose-response profile. At all concentrations, tested from 0.1 to 30 μg/mL, an overall 20% inhibition was observed. Thus, valerenic acid alone is not responsible for the stimulation of GAD activity we previously observed with the *V. officinalis* extract (Awad et al., 2007).

Under the same dose-response conditions, asiaticoside did not inhibit *in vitro* activity even at the highest concentration tested (30 μg/mL) (data not shown). Given that recombinant hGAD65 reaches peak activity at 15 μM PLP

Table 1. Mean percent change (±SD) of *in vitro* hGAD65 activity by phytochemicals and a ginseng extract compared with 3-MPA.

Compound	Percent change	SD
Asiaticoside	4.5	2.1
Betulinic acid	−27.1	10.2
Bilobalide	17.8	6.5
Ginseng extract	−23.0	2.2
Ursolic acid	−11.2	9.6
Valerenic acid	−19.9	4.8
Allylglycine (10 μM)	−2.5	12.6
3-MPA (10 μM)	−43.0	0.3

Each compound was tested at 10 μg/mL in the presence of 15 μM PLP (n = 2 to 3). Positive and negative values represent stimulation and inhibition, respectively.

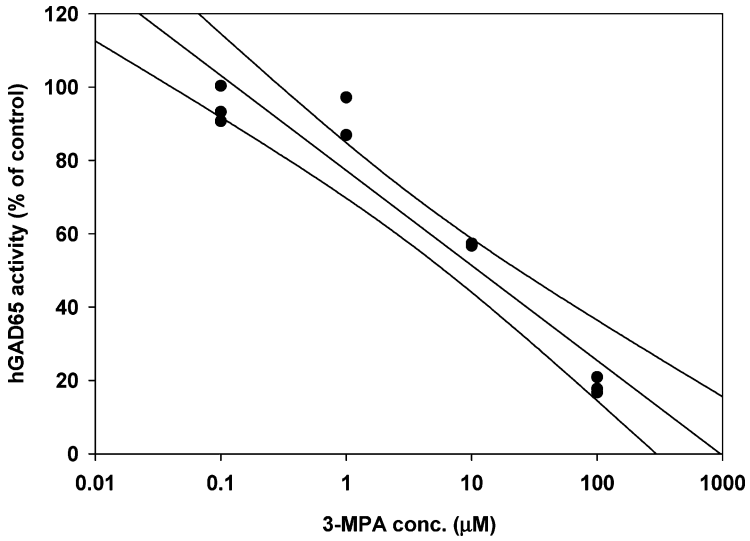


Figure 5. Linear regression analysis (±95% confidence intervals) of the inhibitory effect of 3-MPA on *in vitro* hGAD65 activity, represented as a percent of the control. $IC_{50} = 12.3$ μM (n = 3 for each concentration tested).

(Sardana et al., 2006), enzyme stimulation could potentially be observed if the supplied PLP source decreases. Figure 6 shows that as PLP concentrations increase in the control, the specific enzyme activity of hGAD65 also increases in a dose-dependent manner. Interestingly, at the low dose of 3.75 μM PLP, preincubation with asiaticoside at 10 μg/mL (10.4 μM) significantly stimulated *in vitro* hGAD65 activity by 22% (one-way ANOVA: $p < 0.5$, $F = 13.056$, $N = 8$) compared with the control. There was no statistically significant stimulation of activity in the absence of PLP, nor at the higher PLP doses of 7.5 or 15 μM. From these results, it can be concluded that asiaticoside could be one of the putative stimulatory components of *C. asiatica*. Overall, these experiments demonstrate that the *in vitro* system developed here is capable of detecting both inhibition and stimulation of enzyme activity, two features required for detailed pharmacological assessment.

Discussion

This study reports a new and improved method for producing recombinant human GAD65. To our knowledge, we are the first to design a MBP human GAD65 construct, using the pMAL-c2X vector for expression in *E. coli*. The use of a MBP fusion system allows expression of biologically active recombinant hGAD65 at high levels suitable for pharmacological characterization. This method is also equivalent, if not superior, to the commonly used GST fusion system. The major benefits of the MBP system include faster production time and improved purity of collected fractions.

The goal of this research was to purify hGAD65 for pharmacological evaluation of neuroactive phytochemicals. The safety of many of these compounds is still under investigation, and new techniques are required to assess their biological activity on target enzymes in the brain. Because GAD is responsible for the synthesis of GABA, the main inhibitory neurotransmitter in the CNS, potential interactions with this enzyme should be examined. Generally, GAD65 is more abundant than GAD67 and more concentrated in the synaptic terminals of nerve cells (Martin & Rimvall, 1993; Martin et al., 2000). For this reason, we have begun our analysis with recombinant human GAD65.

The *in vitro* system developed is capable of detecting both enzyme inhibition and stimulation. The GAD inhibitor 3-MPA exhibited a classic dose-dependent response, with

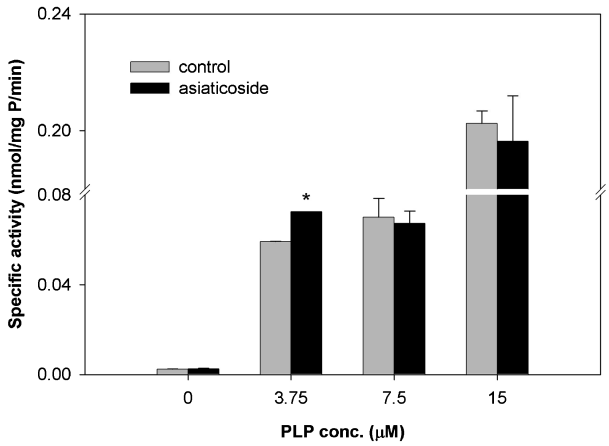


Figure 6. Mean specific activity (nmol mg protein^{1} min^{1}) of hGAD65 *in vitro* in the presence of asiaticoside (10 μg/ml) under varying pyridoxal-5′-phosphate (PLP) concentrations compared with the control (n = 3). Error bars (±SD); *$p < 0.5$.

50% inhibition achieved at approximately 12 μM (Fig. 5). However, when allylglycine was tested at the concentration range between 0.1 μM and 1 mM, no observable effect was noted (Table 1). This result contrasts our initial experiment using rat brain homogenate as a source of total GAD (GAD65 and GAD67). When allylglycine was incubated with tissue homogenate at 1 mM, total GAD activity decreased by approximately 40% ($\pm$4.9) compared with the control. Based on these observations, allylglycine may be a specific inhibitor of GAD67 rather than a general inhibitor of GAD65 and GAD67. Although allylglycine has been shown to inhibit overall GAD activity and decrease GABA levels *in vivo* (Ortiz et al., 1984; Brandao et al., 1986), the exact isoform affected had not been determined. Further studies using a recombinant human GAD67 would clarify these differences and allow for the identification of specific isoform interactions.

Of all the phytochemicals tested, none inhibited hGAD65 to the same extent as 3-MPA. This suggests that these molecules cause limited perturbation in this system. Betulinic acid had the greatest inhibition (27%), followed by the ginsenosides (23%). The inhibition observed with the ginsenosides was unexpected as the same ginseng extract of *P. quinquefolius,* tested at 25 μg/mL in our previous study, stimulated total GAD activity from rat brain homogenates by more than 40% (Awad et al., 2007). The difference in activity shows the importance of using a human enzyme. This is also a phenomenon similar to that of allylglycine, except in the opposite direction. Choi et al. (1998) reported that incubation of purified bovine brain GAD with total ginsenosides from Asian ginseng, *Panax ginseng* C.A. Meyer, resulted in a similar level of stimulation *in vitro*. They also confirmed elevation of GAD *in vivo.* After administration of 50 mg/kg ginsenosides to rats, total GAD activity increased twofold. They further identified the ginsenoside fractions Rb_2 and Rc as the active components. Several differences between the studies should be mentioned. First, the source of ginsenosides tested here was from North American ginseng, *P. quinquefolius* L., and varied slightly in chemical composition. Notably, our ginseng extract had more than 3 times the amount of Rb_1 (59% vs. 18%), which may have been responsible for the variability in activity. Second, Choi et al. (1998) did not differentiate between the two GAD isoforms in their preparation and, presumably, both GAD65 and GAD67 were present. Finally, and perhaps most importantly, we tested purified recombinant human GAD65. Species differences may exist in terms of response to exogenous chemicals, or the ginseng extracts may affect GAD67 to a greater extent. It is clear that in both cases, the ginsenosides are interacting directly with GAD, affecting overall activity, and may therefore modulate GABAergic actions in the brain.

We also found that 10 μg/mL (30.6 μM) bilobalide stimulated *in vitro* hGAD65 activity by 18% ($\pm$6.5) (Table 1). Previous work by Sasaki et al. (1999) showed that bilobalide treatment (30 mg/kg) in mice increased both hippocampal and cortical GABA levels and total GAD activity by about 18%. This amount was sufficient to prevent GABA and GAD reduction induced by the convulsant 4-*O*-methylpyridoxine (Sasaki et al., 2000). The authors proposed that the effect of bilobalide was due to potentiation of GAD, although the exact molecular mechanisms were not clear at the time. Our findings provide more evidence to support this idea and highlight the physiologic importance of small changes in GAD activity.

In the standard assay conditions, asiaticoside did not inhibit hGAD65 activity even at the highest dose of 30 μg/mL. Interestingly, when incubated with limited PLP, there was a significant increase in activity by approximately 20% (Fig. 6). No stimulation was observed in the absence of PLP, so it is unlikely that asiaticoside acts as a PLP mimic. However, it is postulated instead that asiaticoside may interact with the enzyme in a way that facilitates the action of PLP. Further experiments would be required to validate this. The *in vitro* stimulation observed in this study substantiates previous work by Chatterjee et al. (1992), who saw an increase in mouse brain GABA levels after administration of an ethanol extract of *Centella asiatica*.

The enzymatic activity of GAD depends on the cofactor PLP, which binds the specific amino acid sequence Asn-Pro-His-Lys (NPHK) (Qu et al., 1998). The unique physiochemical properties at this active site may influence protein-phytochemical associations. Cysteine residues are also known to play an important role in GAD function (Wu & Roberts, 1974). Similar to lysine 396 on the PLP-binding site, it is believed that the free sulfhydryl group on cysteine 446 in hGAD65 may facilitate Schiff base formation. GAD inhibitors containing a sulfhydryl moiety impair enzyme activity by interacting with cysteine residues, therefore hindering substrate or cofactor binding (Wei & Wu, 2005). It is likely for this reason that 3-MPA is such a potent GAD inhibitor. Although the phytochemicals investigated here do not contain a sulfur group, there may be other active functional groups that could react with critical components of hGAD65. Hydroxyl ($-$OH) groups contain an acidic proton that can interact with a strong base, such as free nitrogen groups on amino acids. Carboxylic acids can also interact with free nitrogen groups, or with $-$OH groups to form esters. The phytochemicals tested possess several of these active groups, thus, binding may have an effect on the conformation or activity of the enzyme. The mechanistic properties of these reactions are speculative at this point, and further investigations about specific phytochemical and protein interactions are warranted.

It can be argued that a minimal change in GAD activity *in vitro* may not translate to significant modifications in a whole organism. Physiologically, however, it is possible that a relatively small change in GAD activity influences brain GABA levels and results in altered neuronal function. For example, early work done by Löscher and Frey (1977) demonstrated that the convulsant picrotoxin at 25 μg/mL inhibited mouse brain GAD activity

in vitro by about 40%. This was followed by *in vivo* experiments, which revealed administration of picrotoxin to mice at 4 mg/kg significantly decreased both total GAD activity and brain GABA concentrations by 15% each. A reduction in GABA at this scale has also been implicated in certain anxiety states such as panic disorder (PD). People suffering from PD have 22% lower GABA levels in the occipital cortex (Goddard et al., 2001) and deficient GABA neuronal response (Goddard et al., 2004). Efforts to find agents that can increase central GABA levels give promise to therapeutics targeting anxiety and related hyperactive nervous disorders. It is important to keep in mind, however, that the regulation of brain function is dynamic. There are many factors other than GAD that are involved in maintaining GABAergic neurotransmission (e.g., GABA transaminase, GABA receptors and transporters, etc.). With respect to the phytochemicals tested in the current study, the inhibitory or stimulatory effects largely depend on the dose and the availability of cofactor. Whether or not these molecules penetrate the blood-brain barrier and directly interact with GAD at these concentrations remain, to be established.

In conclusion, recombinant human proteins represent a new and sophisticated tool for use in pharmacological assessments. This study reports, for the first time, the expression and purification of biologically active human brain GAD65 using the MBP fusion system. The significance of a 20–30% change in GAD activity is physiologically important. Neuroactive phytochemicals that affect GAD activity by a small amount may therefore influence GABAergic function in the brain. The improved method described here holds value in the field of phytopharmacology and can further be used to investigate detailed molecular mechanisms of action.

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