To the Graduate Council :

I am submitting herewith a dissertation written by Yaa Difie Osei entitled "4-Aminobutyrate Aminotransferase: Physical Studies of the Pig Liver Enzyme; Molecular Cloning and Expression of the Human Brain cDNA in Bacterial cells". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfilment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry.

Jorge E. Churchich, Major Professor

We have read this dissertation
and recommend its acceptance :

Accepted for the Council :

Associate Vice Chancellor and
Dean of The Graduate School

4-AMINOBUTYRATE AMINOTRANSFERASE: PHYSICAL STUDIES OF PIG LIVER ENZYME; MOLECULAR CLONING AND EXPRESSION OF THE HUMAN BRAIN cDNA IN BACTERIAL CELLS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

University of Tennessee, Knoxville

YAA DIFIE OSEI

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DEDICATION

This dissertation is dedicated to my husband, **Tim**

and

my children, **Afi**, **Kwabena** and **Asabea**

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ABSTRACT

4-Aminobutyrate aminotransferase (GABA-T) was purified from pig liver using ammonium sulfate precipitation, ion-exchange chromatography and gel filtration techniques. The behavior of the enzyme was studied at acid pH, using analytical gel filtration, circular dichroism (CD) and stopped-flow spectroscopy. The results of gel filtration by FPLC in 50 mM potassium phosphate buffer indicated that the monomeric species predominates, and the quaternary structure was lost at acid pH. The monomers showed no enzyme activity, but activity was restored upon dialysis or dilution at pH 7.0 in potassium phosphate buffer. CD measurements revealed that the secondary structure of the protein remained invariant at both pH 5.0 and 7.0. The kinetics of the dissociation of the dimeric structure at pH 5.0 were characterized by a relaxation time of 18 ms when monitored by stopped-flow measurements of ANS-labeled enzyme; however, the rate of association of the monomeric species at pH 7.0 was too fast to be detected. Based on these results it is postulated that there is an increase in the number of positively charged amino acid residues at acid pH, particularly those arising from the protonation of histidinyl residues. If such residues happen to be at the interface between the subunits, electrostatic repulsion might contribute to destabilization of the quaternary structure.

Fluorescence studies of the ANS-labeled enzyme in the presence of varied concentrations of α-ketoglutarate, one of the substrates for the enzyme, showed that the fluorescence was quenched by the substrate. The removal of at least 7 residues from the COOH-terminal end of the GABA-T by carboxypeptidase Y does not affect its activity.

The cDNA for human brain GABA-T was identified from a human brain cDNA library using a 670 bp *Sma* I/*Kpn* I fragment of the pig brain cDNA for GABA-T. The cDNA was isolated and sequenced, and its tissue distribution was determined. Using the GCG program, an open reading frame consisting of 1503bp encoding a protein of 500 amino acids was predicted, with the first 27 amino acids being assigned to the presequence. Searches of the Genbank revealed a high sequence identity (95.4%) with the rat brain, pig brain and liver enzymes, 48%, for the *A. nidulans* enzyme, 44% for *S. cerevisiae* and 28% for that from *E. coli*.

Hybridization using a 1.46 kbp fragment of the human GABA-T cDNA as a probe for multiple human tissue Northern blots revealed that the detected levels of GABA-T mRNAs were as follows (in decreasing order): liver > pancreas > brain > kidney > heart > placenta, with the transcript being absent in the lung and skeletal muscle, while multiple transcripts were observed for the liver and pancreas. The size of the major transcript in all the tissues was about 5.7 kb.

The cDNA corresponding to the open reading frame was successfully cloned and expressed as a fusion protein using the pET vector expression system (Novagen). The expressed protein was detected by SDS-polyacrylamide gel electrophoresis followed by Coomassie blue staining or Western blots of whole cell lysates and the protein was found to be insoluble. A combination of low temperature incubation for the growth of cells after IPTG induction, solubilization of the protein in urea, purification by affinity chromatography and subsequent stepwise dialysis in decreasing concentrations of urea, in the presence of pyridoxal-5'-phosphate and high concentrations of reducing agent, was used to obtain a soluble protein. The protein preparation showed no enzyme activity. Expression of the protein in the presence of

the co-chaperones, GroEL/ES, likewise, did not result in the production of a soluble protein. It is envisaged that a soluble and active enzyme could be obtained for characterization by manipulating the solubilization parameters or expressing the gene in a mammalian expression system.

TABLE OF CONTENTS

PAGE

INTRODUCTION AND LITERATURE REVIEW 1

EXPERIMENTAL GOALS AND STRATEGIES 13

CHAPTER

1 **PHYSICAL STUDIES OF PIG LIVER 4-AMINOBUTYRATE AMINOTRANSFERASE**

INTRODUCTION 15
EXPERIMENTAL PROCEDURES 16
RESULTS & DISCUSSION 22

2 **ISOLATION, SEQUENCING AND TISSUE DISTRIBUTION OF A cDNA ENCODING THE HUMAN BRAIN 4-AMINOBUTYRATE AMINOTRANSFERASE**

INTRODUCTION 39
EXPERIMENTAL PROCEDURES 40
RESULTS & DISCUSSION 50

3 **MOLECULAR CLONING AND EXPRESSION OF HUMAN BRAIN cDNA ENCODING 4-AMINOBUTYRATE AMINOTRANSFERASE**

INTRODUCTION 65
EXPERIMENTAL PROCEDURES 66
RESULTS & DISCUSSION 74
SUMMARY & FUTURE STUDIES 109

LIST OF REFERENCES 112

APPENDICES 122

VITA 130

LIST OF FIGURES

FIGURE		PAGE
I	The 4-Aminobutyrate Shunt	4
1.1	SDS-PAGE of 4-Aminobutyrate Aminotransferase separated on hydroxyapatite column	24
1.2	Effect of pH on the elution profile of 4-Aminobutyrate Aminotransferase	25
1.3	Circular Dichroism of 4-Aminobutyrate Aminotransferase	27
1.4	Emission spectra of ANS-labeled 4-Aminobutyrate Aminotransferase	29
1.5	Effect of [α-ketoglutarate] on fluorescence intensity of ANS-labeled GABA-T	30
1.6	Fluorescence intensity of ANS-labeled pGABA-T versus [α-ketoglutarate]	31
1.7	Plot of Vo/Vi vs [I]	33
1.8	Dissociation of 4-Aminobutyrate Aminotransferase (Kinetics)	34
1.9	Effect of Carboxypeptidase Y digestion on the catalytic activity of 4-Aminobutyrate Aminotransferase	36
1.10	Thin layer Chromatography of carboxypeptidase Y digested GABA-T	37
2.1	Restriction Enzyme digestion of pET11d+pig brain cDNA insert	51
2.2	Clones in λZAP® II containing GABA-T cDNA after secondary (A) and tertiary (B) screening	52
2.3	A segment of an autoradiogram of a sequencing gel	53
2.4	Nucleotide and predicted amino acid sequences of hGABA-T deduced using the GCG program	54

FIGURE PAGE

2.5 Comparison of the deduced amino acid sequence of hGABA-T with the pig brain enzyme 59

2.6 B*am* HI digestion of pBluescript containing hGABA-T cDNA 60

2.7 Tissue distribution of hGABA-T cDNA by Northern Blot analysis... 61

3.1 B*am* HI digestion of pET30a +/– hGABA-T cDNA established in XL1 Blue 75

3.2 S*al* I digestion of pET30a +/– hGABA-T cDNA established in XL1 Blue 77

3.3 E*co* RI digestion of pET30a +/– hGABA-T cDNA established in XL1 Blue 78

3.4 B*am* HI digestion of pET12a +/– hGABA-T cDNA established in XL1 Blue 80

3.5 S*al* I digestion of pET12a +/– hGABA-T cDNA established in XL1 Blue 81

3.6 E*co* RI digestion of pET12a +/– hGABA-T cDNA established in XL1 Blue 82

3.7 B*am* HI digestion of pET30a +/– hGABA-T cDNA established in BL21(DE3) 83

3.8 E*co* RI digestion of pET12a-hGABA-T +/- GroEL/ES plasmid 85

3.9 Induction time course of hGABA-T fusion protein in BL21(DE3) cells transformed with pET30a-hGABA-T 86

3.10 Induction time course of hGABA-T expressed as S.Tag fusion protein in pET30a followed by Western blotting on whole cell lysate 87

FIGURE PAGE

3.11 Induction time course of hGABA-T expressed as fusion S.Tag protein followed by Western blot on whole cell lysate (L), supernatant (SN) and precipitate (P) 89

3.12 Purification of expressed hGABA-T 93

3.13 Affinity chromatography on His.Bind column followed by SDS-PAGE and Coomassie staining 97

3.14 Western blot analysis used to follow purification of the recombinant GABA-T by affinity chromatography 98

3.15 Solubility of the purified fusion protein in Tris and phosphate buffer..... 99

3.16 Time course of expression after IPTG induction of pET12a +/- hGABA-T in BL21(DE3) cells 102

3.17 Induction time course of expressed hGABA-T in the presence or absence of GroEL/ES 105

3.18 SDS-PAGE following steps for the preparation of crude hGABA-T expressed in the presence or absence of GroEL/ES 106

LIST OF APPENDICES

APPENDIX		PAGE
A1.1	Vector Map for pET30a (Novagen)	123
A1.2	Vector map for pET12a (Novagen)	124
A2.1	Nucleotide and deduced amino acid sequences of hGABA-T showing restriction sites for B*am* HI and S*al* I	125
A3.1	Diagrammatic representation of insertion of hGABA-T cDNA into pET30a in the correct orientation	126
A3.2	Diagrammatic representation of insertion of hGABA-T cDNA into pET30a in the wrong orientation	127
A3.3	Diagrammatic representation of insertion of hGABA-T cDNA into pET12a in the correct orientation	128
A3.4	Diagrammatic representation of insertion of hGABA-T cDNA into pET12a in the wrong orientation	129

LIST OF ABBREVIATIONS

ANS: 1-anilinonaphthalene-8-sulfonate

BCIP: bromo-chloro-indoyl phosphate

bis PLP: P^1P^2-bis (5' pyridoxal diphosphate)

BME: β-mercaptoethanol

CD: circular dichroism

CIP: calf intestinal phosphatase

DEAE: diethylaminoethyl

DTE: dithioerythreitol

DTNB: 5,5' dithiobis(2-nitrobenzoic acid

DTT: dithiothreitol

FPLC: fast performance liquid chromatography

GABA: 4-aminobutyric acid

GABA-T: 4-aminobutyrate aminotransferase

hGABA-T: human brain GABA-T

pGABA-T: pig brain GABA-T

GAD: glutamic acid dehydrogenase

GCG: Genetics Computer Group (Madison, WI, USA)

IAF: 6 iodoacetamide fluorescein

IPTG: isopropyl-β-D thiogalactopyranoside

α-KG: α-ketoglutarate

KPi: potassium phosphate buffer

NBT: nitro blue tetrazolium

ORF: open reading frame

PMSF: phenylmethylsulfonyl fluoride

PLP: pyridoxal-5'-phosphate

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SSA: succinic semialdehyde

SSADH: succinic semialdehyde dehydrogenase

Tris HCl: tris (hydroxymethyl) aminomethane hydrochloride

INTRODUCTION AND LITERATURE REVIEW

The aims of this research are three-fold : first, to evaluate the physical properties of 4-aminobutyrate aminotransferase (GABA-T) at acid pH, and to determine the effect of the removal of COOH terminal residues on the activity of the enzyme; second, to screen a human brain cDNA library for GABA-T cDNA, and sequence it for comparison with sequences from other sources; and third, to express the human brain GABA-T cDNA in *E. coli* cells using the pET expression system in an attempt to provide an abundant and readily available source of the enzyme to facilitate further studies using mutagenesis and chemical modifications to define the mechanism of action of the enzyme. The ultimate goal is to understand more thoroughly the mechanism of action of the human enzyme in order to help in the design and testing of newer drugs for the management of diseases such as epilepsy, in which the action of GABA-T has been implicated.

4-Aminobutyric acid aminotransferase (EC 2.6.1.19) is a mitochondrial matrix enzyme that catalyzes, in the presence of α-ketoglutarate (α-KG), the metabolic breakdown of the inhibitory neurotransmitter, 4-aminobutyric acid (GABA), into glutamate and succinic semialdehyde (SSA). The transamination involves a transfer of the α-amino group of GABA to the α-carbon of α-KG (Bessman, *et al.*,1953 ; Roberts and Bregoff, 1953; Schousboe *et al.*, 1977). Succinic semialdehyde is then converted to succinic acid by another mitochondrial enzyme, succinic semialdehyde dehydrogenase (SSADH). GABA-T belongs to the class of ω-aminotransferases that differ from the majority of mammalian aminotransferases such as aspartate aminotransferase, in that they transfer, in the forward half-reaction an amino group from a carbon atom that does

not bear a carboxyl group, producing an aldehyde instead of a ketone as generally observed for most aminotransferases (Markovic- Housley *et al.*, 1990).

GABA is synthesized from glutamate in an irreversible reaction catalyzed by a cytosolic enzyme, glutamic acid decarboxylase (GAD) (E.C. 4.1.1.15). GAD removes the α-carboxyl group from glutamate, thereby producing GABA and CO_2. It may also be synthesized to some extent by other pathways from metabolites which include ornithine and putrescine (Caron *et al.*, 1987; Daune and Seiler, 1989). Although these pathways may be important in the immature brain or non-neuronal cells (Barres *et al.*, 1990), they do not appear to contribute significantly to the synthesis of GABA in adults. Studies by Fahn and Cote, 1968 and DeFeudis *et al.*, 1970, showed that in the brain of the Rhesus monkey, the highest level of GABA is found in the substantia nigra, globus pallidus and the hypothalamus, with the lowest levels in areas of the brain such as the occipital, frontal and cerebellar cortices. This observation seems to correlate well with the activity of GAD, the enzyme responsible for the conversion of glutamate to GABA, while GABA-T activity is low in the tissues that have high levels of GABA. However, there does not appear to be a consistent inverse relationship between GABA concentration and the transaminase activity (Cooper, *et al.*, 1974).

GABA may serve two functional roles in the brain, with the best characterized function as an inhibitory neurotransmitter, and additionally as an intermediate of energy metabolism. α-KG from the TCA cycle can be converted to glutamate which can be subsequently decarboxylated to give GABA. GABA in turn can be converted to the TCA-cycle intermediate succinate by the actions of GABA-T and SSADH. Both succinate and α-KG are intermediates of the TCA cycle; therefore the entry of these products from the GABA metabolic pathway into the TCA cycle is referred to as the

GABA shunt (Balazs *et al.*, 1970), Fig. I. This route makes it possible for GABA or glutamate to serve as an alternate source of metabolic energy (Baxter, 1970; Sorenson *et al.*, 1991). The mechanisms that regulate GAD and the flux of GABA through the shunt are poorly understood (Martin, 1993). Like other aminotransferases, as well as GAD, GABA-T requires pyridoxal-5'- phosphate (PLP) as a cofactor (Schousboe *et al.*, 1974) for its activity. However, in contrast to GAD, PLP seems to be tightly bound to GABA-T under physiological conditions, since a decrease in the concentration of PLP *in vivo* does not affect its activity (Baxter, 1970; Tapia and Pasantes, 1971). The microenviroment surrounding the cofactor bound to the native enzyme was studied by Felipo *et al.*, (1983) with the aid of ^{31}P-NMR spectroscopy. The results obtained showed that the chemical shift of the phosphorus signal of PLP was pH dependent over the range 6.5 to 7.5, and this was interpreted to mean that PLP was bound in its di-anionic form via a rigid salt bridge to positively charged amino acid residues at the active site, resulting in the lowering of the pKa value compared to that of free PLP in solution (Churchich *et al.*, 1983).

GABA-T has been purified from the brain of mouse (Wu, 1976), human (Cash *et al.*, 1974; White and Sato, 1978), pig (Bloch-Tardy *et al.*, 1974; Beeler and Churchich, 1978), rat (Maitre *et al.*, 1975), guinea pig (Ho *et al.*, 1975) and rabbit (John and Fowler, 1976). In addition to the brain, GABA-T has been found to be present in relatively high concentrations in other mammalian tissues such as the liver and kidney (Tews *et al.*, 1980), and immunological studies have shown that the enzyme from the nervous system is indistinguishable from that of the peripheral tissues (Wu *et al.*, 1978). The enzyme has also been shown to be present in bacterial, yeast and fungal cells (Richardson *et al.*, 1989; Bartsch *et al.*, 1990; André and Jauniaux, 1990), but while the

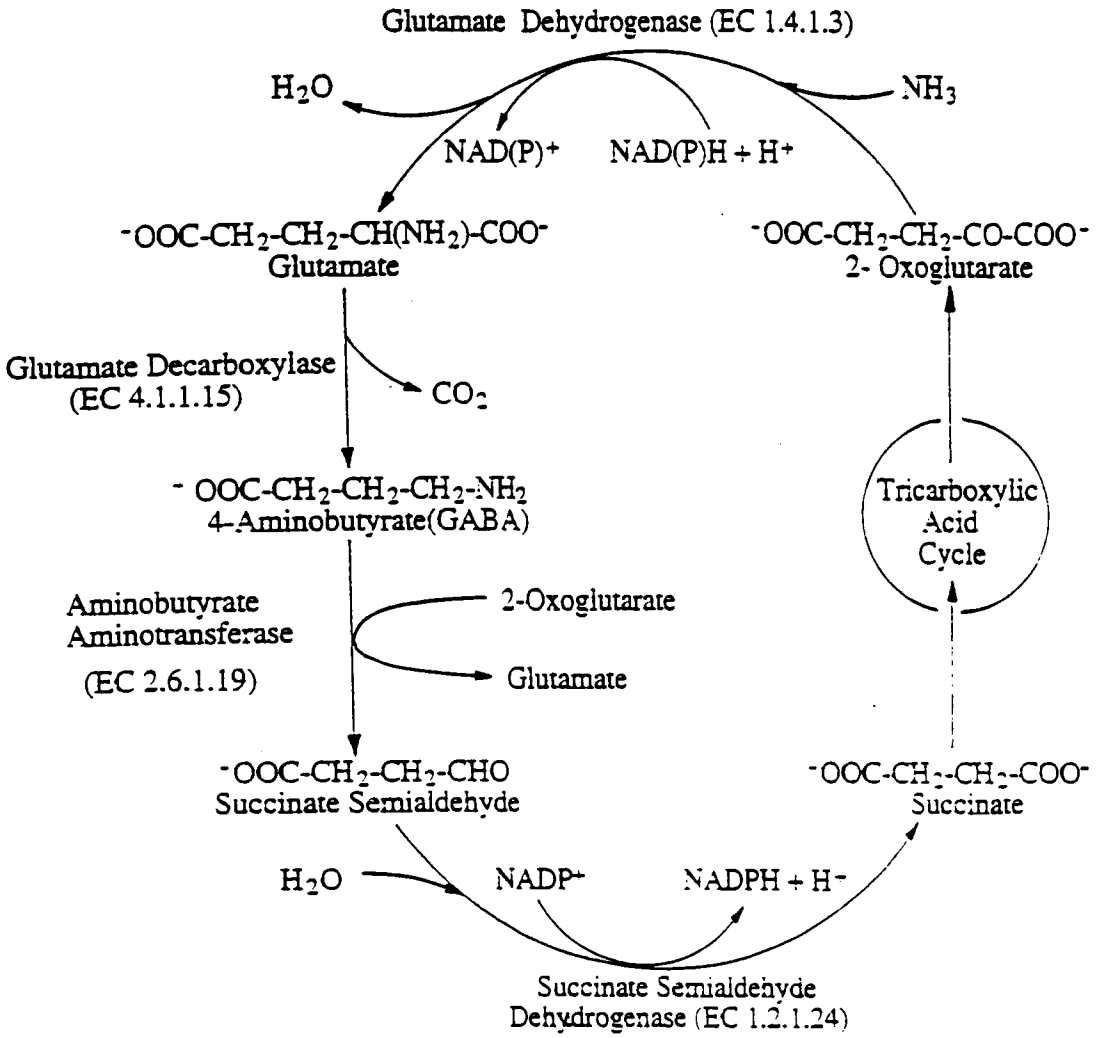


Fig. I: The 4-aminobutyrate (GABA) Shunt

fungal and mammalian GABA-T can act on β-alanine or GABA to the same extent, the bacterial or yeast enzyme is inactive with β-alanine.

The enzyme purified from pig brain and liver is a homodimer with a molecular weight of about 50 kDa per subunit (Bloch-Tardy *et al.*, 1974; John and Fowler, 1976). Buzenet *et al.* (1978), demonstrated that GABA-T could be purified from both the pig brain and liver by almost identical procedures. The native enzyme was stable between pH 6.0-8.5 and over a wide range of protein concentrations from 0.2-4 μM, as demonstrated by fluorescence polarization measurements (Beeler and Churchich, 1978).

A molecule of coenzyme is bound per dimer of GABA-T with a dissociation constant of 10^{-9} M (Churchich and Moses, 1981). Further studies by the same authors showed that a second molecule of the coenzyme could bind less tightly with a K_d of 3 μM, but binding to the secondary site did not result in a change of enzyme activity, as the K_{cat} and K_M values of the enzyme with 1 molecule of PLP/dimer were identical to those for the enzyme with 2 molecules of PLP/dimer. This unusual behaviour was interpreted as reflecting a negative cooperative interaction between the subunits. Inactivation studies with the bifunctional reagent, P',P^2-bis(5'-pyridoxal) diphosphate (bis-PLP), showed that the cofactor was firmly bound to a lysyl residue through a Schiff's base linkage and that bis-PLP blocked other lysyl residues that do not bind the cofactor, but are involved in catalysis (Kim and Churchich, 1982). Out of a total number of 473 amino acid residues for each subunit of the pig brain and liver enzymes, there were 31 lysyl residues with the residue at position 330 identified as the PLP binding residue (Kwon *et al.*, 1992; De Biase *et al.*, 1992). Kinetic studies using the brain enzyme have shown the mechanism of action to be a simple ping-pong Bi-Bi with

the K_M values for GABA and α-ketoglutarate being higher for the brain enzyme than those for the kidney enzyme (Nikolaev and Vasil'ev, 1972; John and Fowler, 1976). Studies with 5,5' dithiobis(2-nitrobenzoic acid) (DTNB) showed that the enzyme was inactivated reversibly in the presence of DTNB. Under denaturing conditions, five sulfhydryl (-SH) groups/dimer were titratable with the reagent. In contrast, another akylating reagent, 6-iodoacetamide fluorescein (IAF), had no effect on catalytic activity and reacted with 3.9 -SH/dimer (Kim and Churchich, 1983). These results implicated the involvement of some specific cysteinyl residues in enzyme activity. Choi and Churchich, (1985) also showed that incubation of GABA-T with iodosobenzoate resulted in the formation of oligomeric species with the subunits cross-linked via a disulfide bond at the interface, rendering the enzyme inactive. These -SH residues were found by Kim and Churchich (1989) to be positioned within 2 Angstroms of each other in a cleft at the interface. Previous studies by Kim and Churchich (1983) indicated that limited proteolysis of GABA-T by trypsin did not impair its catalytic function, and that the catalytic domain remained unperturbed in spite of structural fluctuations detected at the level of the -SH groups.

Role of GABA in neurotransmission

The formation of GABA in the brain was first discovered by Roberts and Frankel (1950), and several authors subsequently demonstrated its role as an inhibitory transmitter in crustaceans (Kravitz *et al.*, 1963; Takeuchi and Takeuchi, 1965; and Otsuka *et al.*, 1966). It has been shown to be the predominant inhibitory neurotransmitter in the inverbrate peripheral nerves and the vertebrate central nervous system (CNS) (Krnjevic and Schwartz, 1967; Roberts, 1976). The physiologic actions of GABA are mediated through two classes of receptors, $GABA_A$ and $GABA_B$. $GABA_A$ receptor is an integral channel protein gating chloride ions, and it consists of

multimeric arrays of α and β subunits (Barnard, 1988). In contrast, $GABA_B$ receptor is a G protein-linked receptor that is negatively coupled to adenyl cyclase, and its activation reduces Ca^+-spike duration and increases K^+ conductance. As a neurotransmitter, GABA binds to the $GABA_A$ receptor and mediates opening of integral chloride channels in the post-synaptic membrane, causing an increase in chloride ion permeability (Olsen, 1982; Glees, 1988). The opening of these channels causes the hyperpolarization to a membrane potential of about -80 mV, preventing any voltage changes that would lead to the propagation of an action potential, and thus inhibiting transmission (Krnjevic, 1974). While GABA acts as an inhibitory neurotransmitter, other neurotransmitters such as acetylcholine act as excitatory neurotransmitters, causing an increase in sodium and potassium ion permeability and thus increasing the membrane potential to about 0 mV, to trigger the propagation of an action potential (Glees, 1988). Glycine is the other major fast inhibitory amino acid neurotransmitter identified in the brain and the spinal cord, while β-alanine, taurine and L-proline are active to lesser extents (Curtis *et al.*, 1968a, 1968b; Aprison and Daly, 1978; Aprison and Nadi, 1978; Davidson, 1976). Glutamate and possibly aspartate are responsible for fast excitatory transmission (Aidley, 1989).

GABA levels and neurological diseases

The production of GABA is very important in the brain since low levels of the enzyme have been associated with a number of neurological disorders, including epilepsy (Tower, 1976), Huntington's disease (Perry *et al.*, 1973) and Parkinsonism (Lloyd *et al.*, 1977). Epilepsy affects about 1-2% of the world's population (Sugarman, 1984; Nanavati & Silverman, 1989) and is broadly defined as any central nervous system disease characterized by recurring convulsive seizures. It has been shown that overactivity of GABA-T or under-production of GABA can precipitate these disorders.

Since GABA synthesis is derived mainly from glutamate by the action of GAD, conditions or agents that inhibit GAD will also affect the GABA concentration. These conditions could be (i) dietary vitamin B6 deficiency, a rare condition which leads to seizures that are promptly relieved by the administration of PLP, (ii) convulsant hydrazides, e.g. hydrazine, which form hydrazones with pyridoxal or PLP, (iii) carbonyl trapping agents such as hydroxylamine which combine with the aldehydic group of PLP, or (iv) structural analogs of vitamin B6, e.g., 4-deoxypyridoxine (Meldrum, 1975).

Although GABA itself does not reach the brain when administered by clinically acceptable routes because of the failure of GABA to cross the blood-brain barrier under normal circumstances (Van Gelder and Elliot, 1958), the observation that epileptic seizures could be terminated by direct injection of GABA into the brain of experimental animals (Hayashi, 1959) showed that anti-convulsant action could be expected if the concentration of GABA in the brain is increased. Some carbonyl trapping compounds such as hydroxylamine and aminooxyacetate were found to inactivate GABA-T, with concomitant increase in the GABA levels, when administered intravenously (Baxter and Roberts, 1961; Kuriyama *et al.*, 1966). Although these compounds demonstrated anti-convulsant properties, they were too toxic to be therapeutically useful as they reacted with other PLP-dependent enzymes (Wood and Peesker, 1973; John *et al.*, 1978). Other compounds which have been designed and tested for GABA-T inactivation are mechanism-based inhibitors, including (i) ethanolamine O-sulfate (Fowler and John, 1972), (ii) a vinyl derivative of GABA, 4-aminohex-5-enoate (Vigabatrin) (Lippert *et al.*, 1977) and (iii) an acetylenic derivative of GABA, 4-aminohex-5-ynoate (Palfreyman *et al.*, 1987). Oral administration of these compounds raised GABA levels by inhibiting the enzyme, but, while the first two compounds were specific for GABA-T with no obvious toxicity, (Leach and Walker, 1977; Phillips and Fowler,1982; Gibson

et al., 1990), the latter compound was toxic as it inactivated other PLP-dependent enzymes (Jung *et al.*, 1977; Jung and Seiler, 1978). Vigabatrin acts as a structural analog of GABA and binds covalently to the active site of GABA-T. When administered in anticonvulsant doses, it increases GABA levels, particularly in the substantia nigra (Harden, 1994). Vigabatrin is one of the most effective anti-epileptic drugs currently in use. More studies are being carried out to design newer anti-convulsant drugs.

Expression of DNA in Heterologous hosts

Manipulation of DNA *in vitro* has developed from the transfer of genetic information between procaryotic organisms (Cohen *et al.*, 1973) to a technology that facilitates efficient and controlled production of proteins in foreign hosts. As the supply of many eukaryotic polypeptides which have potential clinical or industrial use is often limited by their low natural availability, the current techniques for molecular cloning and expression of foreign DNA in these hosts have made it possible to obtain large quantities of some proteins. A prominant example is insulin (Goeddel *et al.*, 1979). Four major expression systems that are commonly available use *E. coli*, *Bacillus subtilis*, yeast and mammalian cells as host cells (Goeddel, 1991). The choice of the type of expression system depends in part on the type of protein and the intended use for the protein after expression. For example, if the protein is small (~ 80 amino acids), it may be easily expressed as a fusion protein in *E. coli*. For polypeptides ranging from 80 to 500 amino acids which are normally secreted proteins, e.g. enzymes and hormones, any of the four systems listed could and have been used for expression. For very large (greater than 500 amino acids) secreted proteins and surface receptor proteins, success has been mostly limited to mammalian expression systems. If the purpose for expressing a new gene or cDNA is to verify that the correct sequence has

been isolated, then transient expression in mammalian cells is often preferred, particularly if the cloned protein is from a higher eukaryote since expression in similar hosts promises a high probability of yielding biologically active protein. If on the other hand, the main reason for the expression is to produce large quantities of antigen for antibody generation, then the expressed protein does not have to be correctly folded and the *E. coli* system could be used. In addition, if the goal in expression of the cloned gene is to obtain mutagenized protein for structure-function experiments, then the simplest expression system in this case is *E. coli* since it is easy to generate many defined mutants in such a system. As some proteins have to be modified post-translationally to give an active protein, it is imperative that the expression system selected should mimic the natural source of the protein as closely as possible.

Although yeast and *B. subtilis* are not usually the first choice for expression of heterologous genes, they have certain advantages that make them good candidates for some specific proteins. The greatest advantage of *B. subtilis* is its ability to secrete proteins directly into the culture medium, where they accumulate to high levels in a relatively pure state and very often correctly folded, disulfide-bonded and soluble form. The disadvantage with this system is that the efficiency of secretion of the protein cannot be predicted, as some proteins are efficiently secreted, while others closely related are not (Henner, 1991). Yeast cells offer the advantages that they can be propagated in minimal media and have a complex secretory pathway, which allows them to secrete the foreign protein into the culture medium for easy purification. In addition, since they are eukaryotes, they can carry out post-translational modifications required for some proteins (Emr, 1991). The mammalian expression system produces stable proteins, but has the disadvantage of being time-consuming in selection, amplification and the growth of stable cell lines (Goeddel, 1991). In spite of the

advantages of the other expression systems, the *E. coli* expression system has been the choice for the expression of many heterologous genes and as such several systems have been developed for the cloning and expression of recombinant proteins in *E. coli* with one of the most powerful being the pET System. This system was designed based on the T7 promoter-driven system originally developed by Studier and Moffat, (1986), Rosenberg *et al.*, (1987) and Studier *et al.*, (1990) and now with newer derivatives designed with enhanced features for subcloning, detection and purification of target proteins developed by Novagen (pET System Manual, Novagen). For this system, target genes are cloned under the control of strong bacteriophage T7 transcription and translation signals and expression is induced by addition of IPTG. The T7 RNA polymerase is very selective and directs most of the resources of the cell to the expression of the target gene, resulting sometimes in the target protein forming as much as 50% of the total cellular protein after few hours of induction. In practice, the target gene is cloned into the pET expression vector and initially established in non-expression host *E. coli* K12 strains such as NovaBlue, HB101, and XL1-Blue which do not contain the T7 RNA polymerase gene, so that background production of potentially toxic proteins that can cause instability of the plasmid is minimal. In addition to this, these host cells are $recA^-$ and give high transformation efficiencies and good plasmid yields. Once the plasmids are established, they are transferred into λDE3 lysogens of either bacterial strain BL21 or HMS174 expression hosts containing a chromosomal copy of the T7 RNA polymerase gene under *lac*UV5 control, and expression is induced by addition of IPTG.

Another property of the pET vectors that makes it very convenient in their use in expression is the presence of different sequences adjacent to the cloning sites that code for a variety of peptide "tags" for the detection and purification of the target protein.

The S-Tag sequence in pET 30, for example, codes for a 15 amino acid peptide of pancreatic ribonuclease A. Advantage is taken of the interaction of this peptide with the 104 amino acid S-protein derived from pancreatic ribonuclease A for the detection of the S-Tag fusion protein by Western Blot. Blots are prepared and incubated with the S-Protein Alkaline Phosphatase Conjugate and the target bands visualized using the NBT/BCIP substrate. The His-Tag sequence found in some of the vectors, e.g. pET30, encodes a consecutive stretch of 6 or 10 histidine residues that can be expressed as part of the N-terminal and/or C-terminal end of the target protein. The His-Tag peptide sequence binds to divalent cations (Ni^{2+}) immobilized on the His-Bind resin (Van Dyke *et al.*, 1992). S-Tag fusion proteins produced from appropriate vectors can be incubated with S-protein covalently bound to agarose beads and the target protein released by incubation of the agarose beads with biotinylated thrombin, leaving the S-Tag peptide bound to the resin. This means that both the S-Tag and His-Tag peptide sequences can be used in affinity chromatography for the purification of the target protein. The choice of pET 30a (Appendix A1.1) for expression therefore provides for both easy identification and purification since it has both "Tags". In addition, some of the pET vectors, e. g. pET12 (Appendix A1.2), allow the fusion of the target DNA to the N-terminal signal sequence of the vector for potential periplasmic localization, thus preventing its accumulation in the cytoplasm which may result in either aggregation or degradation.

EXPERIMENTAL GOALS AND STRATEGIES

For the physical studies using pig liver GABA-T, the behavior of the enzyme at acid and neutral pH was explored to help in understanding the structural properties of the enzyme. Some of the questions posed included the following:

(i) Is the enzyme intact as a dimer with a decrease in pH?

(ii) Is the monomer catalytically active?

(iii) What is the secondary structure of the monomer?

(iv) What is the rate of association/dissociation?

In order to answer these questions analytical gel filtration, circular dichroism, fluorescence and stopped-flow spectroscopy were employed. Since the enzyme has been shown to be a homodimer with no interchain disulfide bonds, it was envisaged that the monomers could be separated using gel filtration if proper conditions were defined to promote dissociation. Circular dichroism measurements in the far-UV region, indicative of the secondary structure of proteins, were employed to determine any changes in the secondary structure of GABA-T at acid pH. In addition, changes in the microenvironment around the PLP-binding site were assesed by measuring CD in the near-UV region since PLP bound to the intact enzyme has a characteristic spectrum in this region. Comparison of the CD spectra of the enzyme in buffer of differing pHs should give an indication of any structural changes which occur as a function of pH. Stopped-flow measurements were used to follow the kinetics of dissociation.

The second part of this research was aimed at expressing the cDNA of human brain GABA-T in bacterial cells. Since it is necessary to have the enzyme available in large quantities for testing and developing new drugs with anti-convulsant action aimed

at inhibiting GABA-T activity in humans, it would be advantageous to have a form of the enzyme which can be over-expressed. Since the cDNA for the pig brain GABA-T had been isolated in this lab, a fragment of this cDNA was used as probe to screen a human brain cDNA library for the GABA-T cDNA. In an attempt to express the enzyme, the isolated cDNA was cloned into vectors allowing expression in *E. coli* cells under strict control of the T7 promoter.

CHAPTER 1

PHYSICAL STUDIES OF PIG LIVER 4-AMINOBUTYRATE AMINOTRANSFERASE

INTRODUCTION

4-Aminobutyrate aminotransferase (GABA-T), the enzyme that catalyzes the breakdown of the major inhibitory neurotransmitter, 4-aminobutyrate, has been purified from a number of sources including rat brain (Bloch-Tardy *et al.*, 1971), pig brain (Churchich and Moses, 1981) and liver tissues (Buzenet *et al.*, 1978). The enzyme appears in all cases to be a dimer consisting of two identical subunits of molecular weight about 50 kDa/monomer. One molecule of PLP is required per dimer for catalytic activity. Although numerous studies have addressed the structure/function relationships of the active dimeric enzyme, the structure and function of the monomeric form have not been examined in detail. In addition, it has also been shown that limited proteolysis by trypsin does not impair the catalytic activity of the enzyme, with the enzyme remaining as a dimer (Kim and Churchich, 1983). In this thesis, attempts were made to characterize the enzyme at acid pH using physical methods such as analytical gel filtration to determine whether the dimeric structure was intact at that pH, circular dichroism and fluorescence measurements to detect any changes in the secondary and tertiary structures, respectively, and stopped-flow spectroscopy to measure the kinetics of dissociation/association. The proteolysis from the COOH terminus of the enzyme was carried out using carboxypeptidase Y to determine the extent of cleavage by the protease and the effect of the cleavage on the enzyme activity.

Results from this study show that the enzyme is dissociated at acid pH to give monomers which are catalytically inactive. The secondary structure is intact, and the dissociation is reversible, with enzyme activity restored upon association of the monomers. The removal of at least 7 amino acid residues from the COOH-terminal does not impair the catalytic activity.

EXPERIMENTAL PROCEDURES

Materials

Pig liver was obtained from Swaggerty Sausage Company, Kodak, TN. Resins for chromatography, i.e., carboxymethyl-Sephadex C-50, DEAE-Sephadex and Superose 12, were purchased from Pharmacia LKB Biotechnology, Inc. Hydroxylapatite was prepared following the procedure by Levin (1962). 1-Anilinonaphthalene-8-sulfonate (ANS), 4-aminobutyrate (GABA) and α-ketoglutarate (α-KG) were from Sigma Chemical Company, St Louis. Missouri. All other reagents used were of analytical grade.

Preparation of GABA-T from pig liver

The liver was obtained as soon as the animal was slaughtered and quickly chilled on ice for transportation. The enzyme preparation was carried out following the method of Churchich and Moses, 1981. Unless otherwise stated, all the purification steps were performed at 4°C and all the buffers used contained 0.1 mM EDTA and 1 mM β-mercaptoethanol. A 25% (w/v) homogenate was prepared by homogenizing the liver in ice-cold 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM α-ketoglutarate in a Waring blender. The resulting homogenate was adjusted to pH 5.5 by

slow addition of 1M acetic acid, heated to 55°C, for 5 min, kept on ice in the cold room with occasional stirring and allowed to cool to 4°C. The homogenate was centrifuged at 10,000 x g at 4°C for 30 min and the precipitate discarded. The supernatant was treated with ammonium sulfate and the precipitate obtained at 40-75% saturation was redissolved in 0.01 M sodium acetate buffer, pH 5.7 (buffer I) and dialyzed overnight against several changes of the same buffer. The sample was centrifuged at 10,000 x g for 20 min and the supernatant applied onto a CM-Sephadex column (5 x 80 cm) previously equilibrated with buffer I. The column was washed with the same buffer (5 x column volume) and the enzyme eluted by using a linear gradient made up of buffer I (500ml) and the same volume of 0.3 M sodium acetate buffer, pH 5.7. The column was washed with another 250ml of 0.3 M sodium acetate buffer, pH 5.7 at the end of the gradient to ensure complete elution of the enzyme. The fractions showing activity were pooled and concentrated using an Amicon concentrator (membrane size, PM10), and the concentrated sample was dialyzed against 0.01 M potassium phosphate buffer, pH 7.4 (buffer II).

The sample was applied to a DEAE-Sephadex column (2.6 x 30 cm), previously equilibrated with buffer II, washed with 100 ml buffer II and eluted using a linear gradient made of buffer II (250 ml) and the same volume of 0.2 M potassium phosphate buffer pH 7.4. The active fractions were pooled, concentrated to about 50 ml and dialyzed against 0.01 M potassium phosphate buffer, pH 7.0 without EDTA (buffer III). The sample was applied to a hydroxylapatite column, (0.9 x 20 cm), previously equilibrated with buffer III, washed with 50 ml of the same buffer and the enzyme eluted with a linear gradient made of buffer III (50 ml) and the same volume of 0.35 M potassium phosphate buffer, pH 7.0. All the fractions showing activity were pooled, dialyzed against several changes of 50 mM potassium phosphate buffer, pH 7.0 and

stored at -20°C in small aliquots and used for subsequent studies. The purity of the enzyme preparation was determined by running a sample on SDS- polyacrylamide gel and the protein bands stained with Coomassie blue stain R250. Protein concentration was determined by the Bradford method (Bradford, 1976).

Enzymatic Assays

Two different methods were used in the assay of GABA-T activity. One was a coupled assay system consisting of the purified GABA-T and succinic acid semialdehyde dehydrogenase (Churchich and Moses,1981). The dehydrogenase was obtained by treating commercially available GABASE (Sigma), a combination of GABA-T and the dehydrogenase, with hydroxylamine to destroy the GABA-T, and the excess hydroxylamine was removed by extensively dialyzing the treated enzyme against several changes of 50 mM potassium phosphate buffer, pH 7.0, containing 2 mM EDTA and 1 mM β-mercaptoethanol. Enzymatic assays were performed in 0.1 M sodium pyrophosphate buffer, pH 8.4, containing 5 mM $NADP^+$, 20 mM 4-aminobutyrate and 10 mM α-ketoglutarate. The progress of the reaction was followed by monitoring changes in absorbance at 340 nm due to the reduction of $NADP^+$ within three minutes.

The other assay method is based on fluorometric measurements of the condensation product of cyclohexane 1,3-dione with succinic semialdehyde (Beeler and Churchich, 1978). The substrate solution for the assay, was 20 mM potassium phosphate buffer containing 20 mM 4-aminobutyrate and 10 mM α-ketoglutarate, adjusted to pH 7.0 with NaOH; the reagent solution was prepared by the addition of 0.25 g of cyclohexane 1,3-dione, 10 g of ammonium acetate and 5 ml of glacial acetic acid in a total volume of 100 ml with deionized water. The substrate solution (5 ml) was incubated with the enzyme at 37°C and 1 ml aliquots were withdrawn at several

time intervals, mixed with 0.1 ml reagent solution and incubated in a water bath at 60°C for 15 min. The reaction was stopped by addition of 1 ml deionized water, and the fluorescence intensity of the condensation product was recorded at 460 nm, with excitation at 360 nm. A control reaction was set up without any enzyme.

Gel Filtration Chromatography

The purified enzyme (1 mg) was chromatographed by fast performance liquid chromatography (FPLC) at 10°C on a Superose 12 column (480 x 16 mm) (particle size 20-40 μm) at a flow rate of 0.5 ml/min in a Pharmacia instrument. The mobile phase was either 50 mM potassium phosphate buffer pH 7.0 or pH 5.0. Standards of known molecular masses were chromatographed on the column using the same buffers. The standards were thyroglobulin (670 kDa), gamma-globulin (158 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa).

Circular Dichroism (CD) Measurements

CD measurements were recorded on a Jasco (J-40A) spectropolarimeter using a 1 cm cell. CD spectra covering the range 210-250 nm and above 350 nm were acquired using protein concentrations ranging from 0.02 to 0.1 mg/ml and 2 to 5 mg/ml, respectively, in 50 mM potassium phosphate buffer pH 5.0 and 7.0. The spectrometer was routinely calibrated with the asymmetric compound, (+) 10-camphorsulfonic acid (Compton and Johnson,1986).

Ellipticity, [θ], expressed in terms of mean residue ellipticity was calculated with the aid of the following equation (1):

$$[\theta] = \theta \times MRW/10 \times l \times c. \qquad (1)$$

where θ is ellipticity in mdeg; MRW, Mean Residue Weight of an amino acid; l, pathlength in cm; and c, concentration in g/ml.

Fluorescence Measurements

Fluorescence measurements were conducted in a Perkin-Elmer LS-50B spectrofluorometer on GABA-T, upon titrating with increasing concentrations of 1-Anilinonaphthalene-8-sulfonate (ANS). The samples were excited at 360 nm and the fluorescence enhancement at 480 nm recorded. The titrations were performed in 50 mM potassium phosphate buffer at pH 5.0 and 7.0, using a fixed concentration of GABA-T (4 μM) to determine the stoichiometry of binding. The results were analyzed using the following equation by Scatchard, *et al.*, (1957)

$$\upsilon/[L] = K(n-\upsilon) \qquad (2)$$

where υ is the average concentration of ANS bound to the enzyme; [L], the concentration of free ligand; K, the equilibrium constant; and n, the number of binding sites. An extinction coefficient of 4.9×10^3 $M^{-1}cm^{-1}$ was used for the free and bound ANS.

Effect of α-ketoglutarate on binding of ANS

GABA-T (5 μM) was incubated with ANS (50 μM) in 20 mM potassium phosphate buffer, pH 7.0 and aliquots of (1 M) solution of α-ketoglutarate prepared in the same buffer with the pH adjusted to 7.0 using NaOH, were added. The fluorescence intensities were recorded between 400-600 nm upon excitation at 360 nm. Measurements were recorded for increasing concentrations of α-ketoglutarate.

Stopped-flow Measurements

The dissociation of GABA-T at pH 5.0 was studied using the Bio-logic Stopped-flow SFM:3 System. For kinetic measurements, the protein (50 μM) in the presence of ANS (400 μM) in 20 mM potassium phosphate buffer, pH 7.0, was mixed with a 10 fold excess volume of 50 mM potassium phosphate (pH 4.2) and the increase in fluorescence at 480 nm was immediately recorded upon excitation at 360 nm.

Carboxypeptidase Y Digestion of GABA-T

The digestion of GABA-T with carboxypeptidase Y was carried out by incubating 3 ml GABA-T at a concentration of 2 mg/ml previously dialyzed against 0.1 M ammonium bicarbonate, pH 7.4, with carboxypeptidase Y in the ratio 50 : 1. The mixture was incubated at either 25°C or 37°C for up to three hours and aliquots withdrawn at different time intervals for enzyme activity measurements. At the end of the incubation period the protein was precipitated and the supernatant carefully transferred into a clean tube. Aliquots were applied together with amino acid standards onto thin layer chromatography plates and resolved using n-butanol/pyridine/acetic acid/H_2O in the ratio, 15 : 10 : 3 : 12. The spots were developed by spraying with 0.01% fluorescamine in acetone, dried and viewed under UV light. The remaining supernatant was lyophilized and used for amino acid analysis which was kindly performed at the University of Tennessee Medical Center, Knoxville.

RESULTS AND DISCUSSION

Purification of pig liver GABA-T:

Table 1 shows the scheme of GABA-T purification. The enzyme was eluted at a concentration of 0.3 M sodium acetate on CM-Sephadex column and at phosphate concentrations of 0.05 M and 0.28 M on DEAE-Sephadex and hydroxylapatite columns, respectively. The final purification step on hydroxylapatite was to free the enzyme from aspartate aminotransferase which has been shown to contaminate GABA-T during the purification on DEAE-Sephadex column. The yield was 10% with a specific activity of 0.17 μmol/mg/min. The purified enzyme was > 90 % pure when detected by Coomassie blue staining after SDS-polyacrylamide gel electrophoresis (Fig. 1.1). The purer fractions in lanes 1 & 2 were used for most of the study.

Gel-Filtration Chromatography:

Fig. 1.2, illustrates the elution profiles obtained when the purified enzyme was chromatographed by FPLC on a Superose column in 50 mM potassium phosphate buffer, pH 5.0 and 7.0. From the profile it could be seen that the enzyme elutes at a time corresponding to a molecular weight of 52 kDa at pH 5.0 when compared with the profile for the standards. At pH 7.0, however, the enzyme elutes at a time corresponding to the molecular weight of the dimeric form, 105 kDa. This indicates the enzyme dissociates at acid pH. When the enzyme was incubated at 25°C for 30 min in 50 mM phosphate buffer, pH 5.0 and extensively dialyzed in phosphate buffer, pH 7.0, and aliquots taken or directly diluted in assay mixture containing the substrates GABA and α-KG at pH 8.4, full catalytic activity was recovered. No catalytic activity was observed when the reaction was carried out at acid pH using the fluorometric method (Beeler and Churchich, 1978). These observations were interpreted to mean

Table 1. Purification of 4-aminobutyrate aminotransferase from pig liver.

The starting material was approximately 5 kg.

Purification step	Volume (ml)	[Protein] (mg/ml)	Specific Activity at 25°C (nmoles/mg.min)	Total Protein (mg)	Total Activity (nmoles)	Yield (%)
Crude homogenate	4800	149	0.228	715,200	163,066	100
Ammonium sulfate	1000	107	1.03	107,000	110,210	68
CM-Sephadex	180	49.1	9.9	8,838	87,496	54
DEAE-Sephadex	100	14.2	24	1,420	34,080	21
Hydroxylapatite	30	3.1	170	93	15,810	9.7

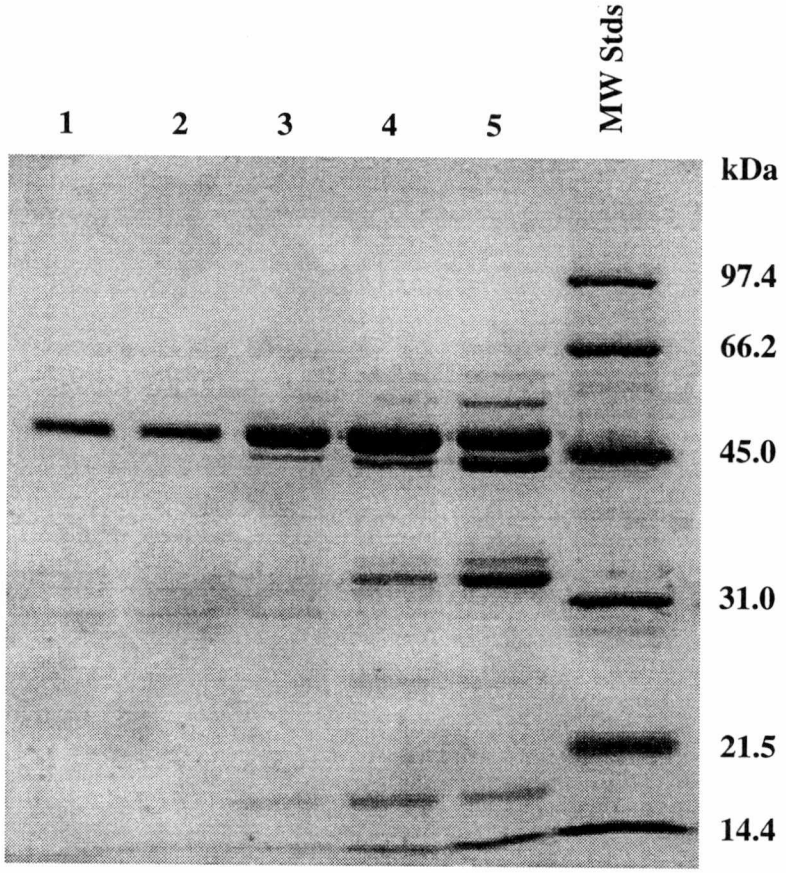


Fig 1.1 : SDS-PAGE of 4-aminobutyrate aminotransferase separated on hydroxylapatite column.

Pooled sample with GABA-T activity after DEAE-Sephadex column chromatography was dialyzed in 0.01M potassium phosphate buffer pH7.0 and applied to a hydroxylapatite column equilibrated in the same buffer. The enzyme was eluted by using a linear gradient made of equal volumes of 0.01M and 0.35M potassium phosphate buffer pH7.0. The enzyme eluted at phosphate concentration of 0.28M. Eluted fractions showing GABA-T activity were pooled into four separate fractions and aliquots (10μL) of each fraction applied to SDS-PAGE.

Lanes 1-4 - Pooled fractions with GABA-T activity
5 - Initial sample applied to the column

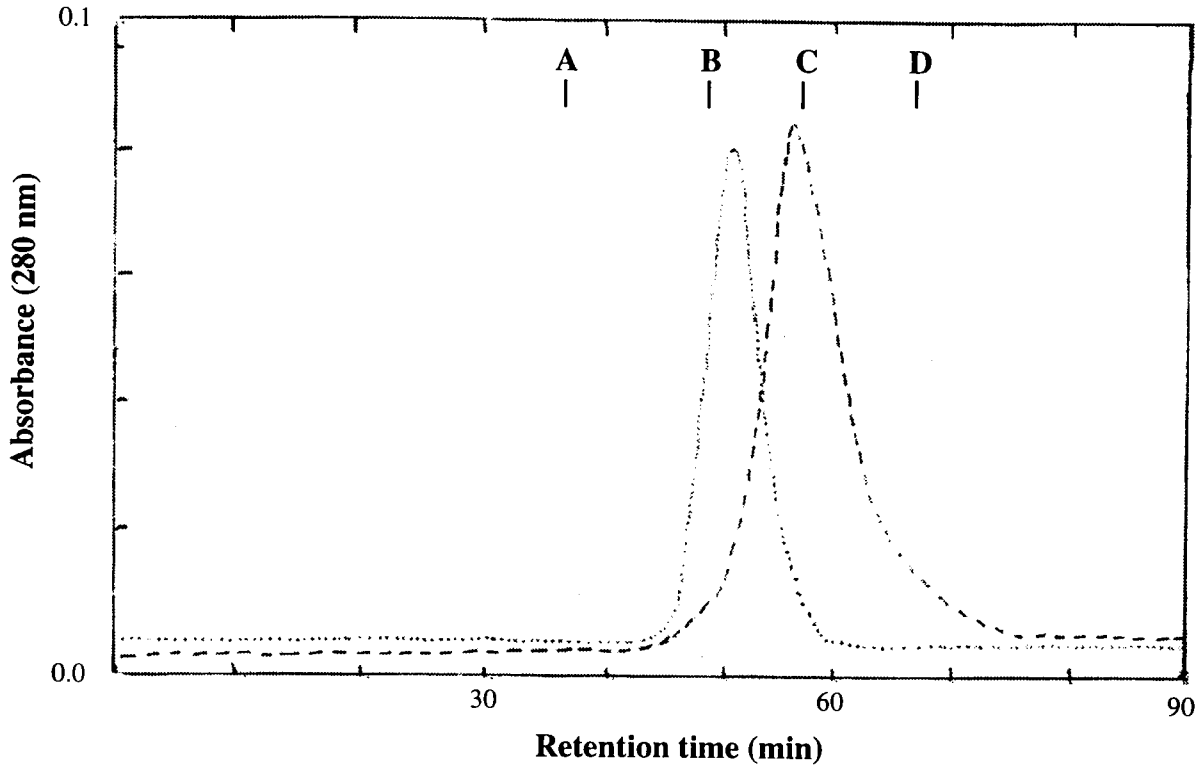


Fig. 1.2 : Effect of pH on the elution profile of 4-aminobutyrate aminotransferase

The enzyme (1 mg) was chromatographed on a Superose 12 column (480x16 mm) at a flow rate 0.5 ml/min at 10°C. Elution of the enzyme when the mobile phase was 50 mM potassium phosphate (pH 7.0) (...) and 50 mM potassium phosphate (pH 5.0) (---). Standards of known molecular weights were chromatographed under similar conditions. A,thyroglobulin (670 kDA); B, γ-globulin (150 kDA); C, ovalbumin (44 kDa); and D, myoglobin (17 kDA).

that the dimeric form of the enzyme dissociated at acid pH into monomers which had no catalytic activity, and that the dissociation was reversible.

The amino acid sequences of both pig brain and pig liver enzymes indicate the presence of 14 histidinyl residues (Kwon *et al.*, 1992; De Biase *et al.*, 1992) with an isoelectric point of 7.25. At acid pH, histidines would be protonated and there would be a net increase in positive charge on the protein. It is postulated that electrostatic repulsion between positively charged residues at the interface of the subunits may contribute to the destabilization of the quartenary structure and result in the dissociation of the dimer.

CD Measurements

Fig. 1.3A shows the CD spectra recorded at 210-250 nm for the enzyme in 50 mM potassium phosphate buffer, pH 5.0 and 7.0. The ellipticity remained invariant at both pHs, implying that there were no measurable differences in the secondary structure for the protein, as the far-UV region of the CD spectrum provides a qualitative measure of the average secondary structure content of a protein (Johnson, 1988). Fig. 1.3B shows the CD spectrum of the bound PLP when the protein was examined between 300-450 nm in 50 mM phosphate buffer, pH 5.0 and 7.0. Free PLP is inherently symmetrical and therefore an optically inactive chromophore, yet its binding to the active sites of enzymes usually generates induced optical activity which can be recorded by CD (Kallen *et al.*, 1985). As observed in the case of aspartate aminotransferase, PLP bound to the catalytic site of GABA-T displays two positive CD bands at 360 and 445 nm and two negative ellipticity bands at 330 and 415 nm. Again, the spectral shape and magnitude of the ellipticity values remained invariant at both acid and neutral pH values, implying that the microenvironment surrounding the cofactor was not affected

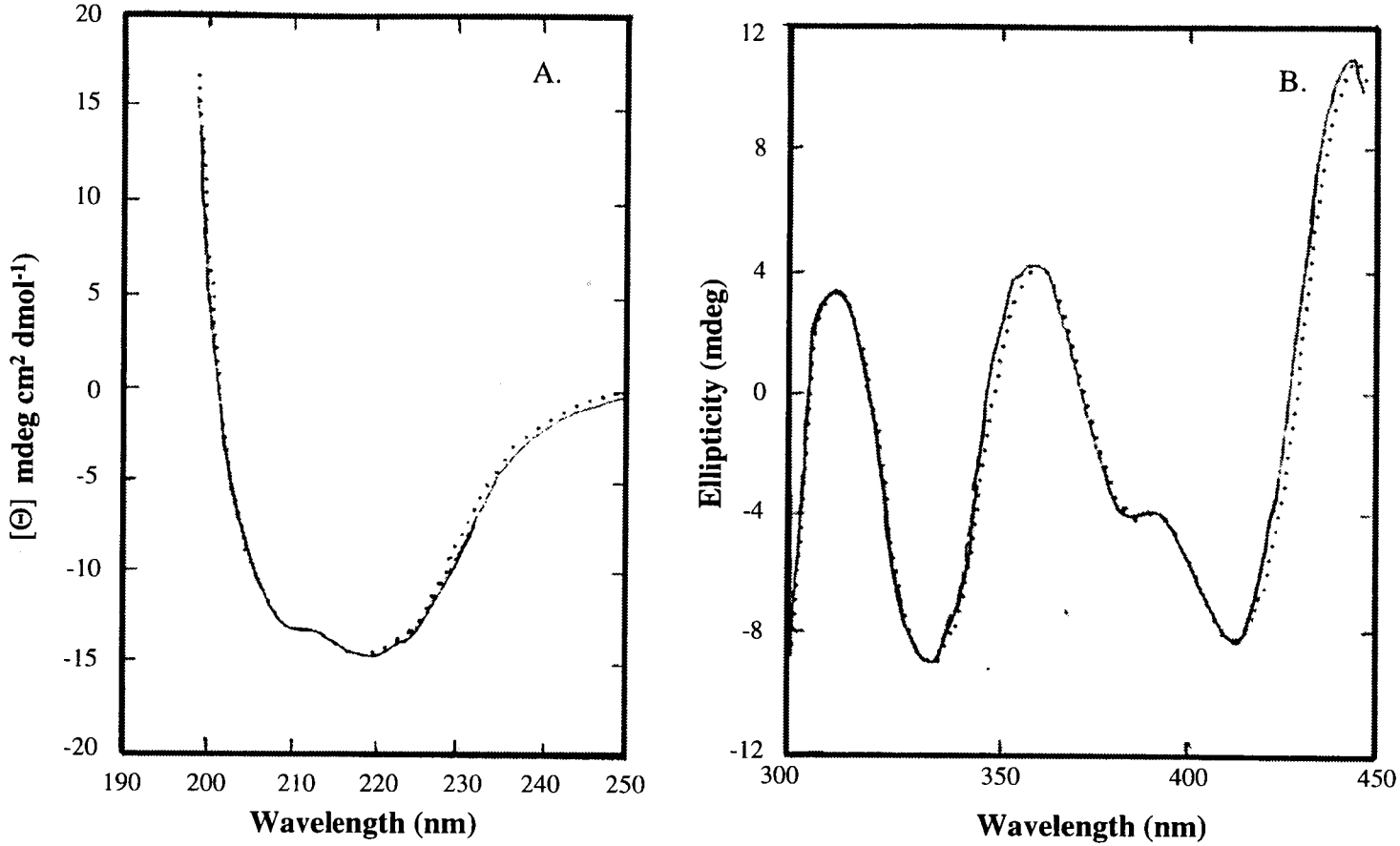


Fig. 1.3 : Circular Dichroism of 4-aminobutyrate aminotransferase

A. Plot of the ellipticity [mdeg cm^2 $dmol^{-1}$] of samples of 4-aminobutyrate aminotransferase (0.1 mg/ml) at pH 7.0 (---) and pH 5.0 (....)

B. Ellipticity (mdeg) of samples of 4-aminobutyrate aminotransferase recorded over the spectral range 300-450 nm at a protein concentration of 3 mg/ml. Identical CD spectra were observed for samples examined at pH 5.0 (...) and pH 7.0 (---)

by the state of association or dissociation of the enzyme. The CD results obtained could be interpreted to mean that the secondary structure was not affected and remained intact, although a decrease in pH caused loss of the quaternary structure.

Exposure of Hydrophobic Binding Sites

When ANS molecules bind to non-polar sites on a protein, there is an increase in fluorescence intensity as well as a blue shift in the emission maximum (Laurence, 1952). This property of ANS was exploited to monitor the formation of hydrophobic sites during the dissociation of the dimeric enzyme. The magnitude of the fluorescence enhancement at 480 nm was shown to be dependent on the pH of the medium, as shown in Fig. 1.4. At pH 5.0, there was an increase in the fluorescence intensity compared to the value at pH 7.0, with the ratio of the bound to free ANS at pH 5.0 equal to 15 and that at pH 7.0 equal to 10. The difference in fluorescence yield reflects that more hydrophobic sites had been made available for ANS to bind at acid pH. In the absence of the protein however, the intensity of fluorescence of ANS was the same at pH 5.0 and 7.0. There was a blue shift (from ~500 nm to 480 nm) when the label bound to the protein at either pH, a characteristic property observed for ANS binding to macromolecules (Lakowicz, 1983). It may be deduced from these observations that some hydrophobic residues which were buried in the dimeric state had been exposed upon dissociation.

Inhibition Studies of ANS-labeled GABA-T by α-KG

The results of fluorescence studies with ANS-labeled GABA-T in the presence of increasing concentrations of α-ketoglutarate are shown in Figs. 1.5&1.6. The λ_{max} of emission of ANS shifted from 510 nm to 480 nm upon binding to the protein, reflecting the binding of ANS at a more non-polar environment. However, upon

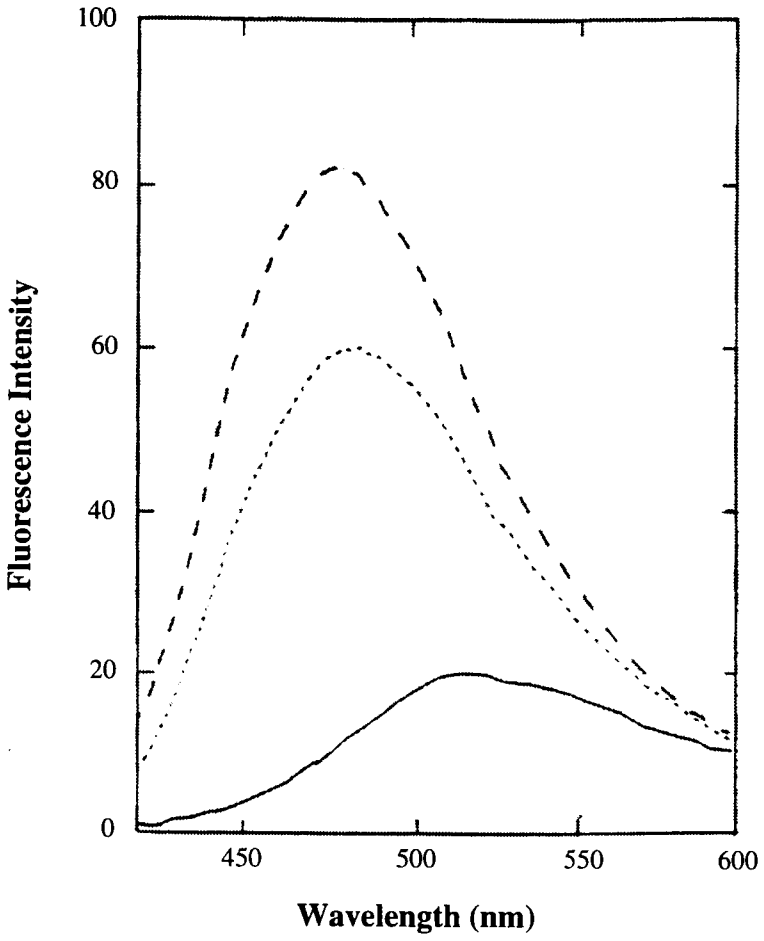


Fig. 1.4 : Emission spectra of ANS-labeled 4-aminobutyrate aminotransferase

Emission spectra of free ANS (5 μM) at pH5.0 and pH7.0 (___)
ANS (5 μM) + enzyme (2.5 μM) at pH5.0 (---) and pH7.0 (...) 50 mM potassium phosphate. Excitation wavelength was 360 nm. The samples were allowed to incubate at room temperature (25°C) for 30 minutes before recording the emission spectra.

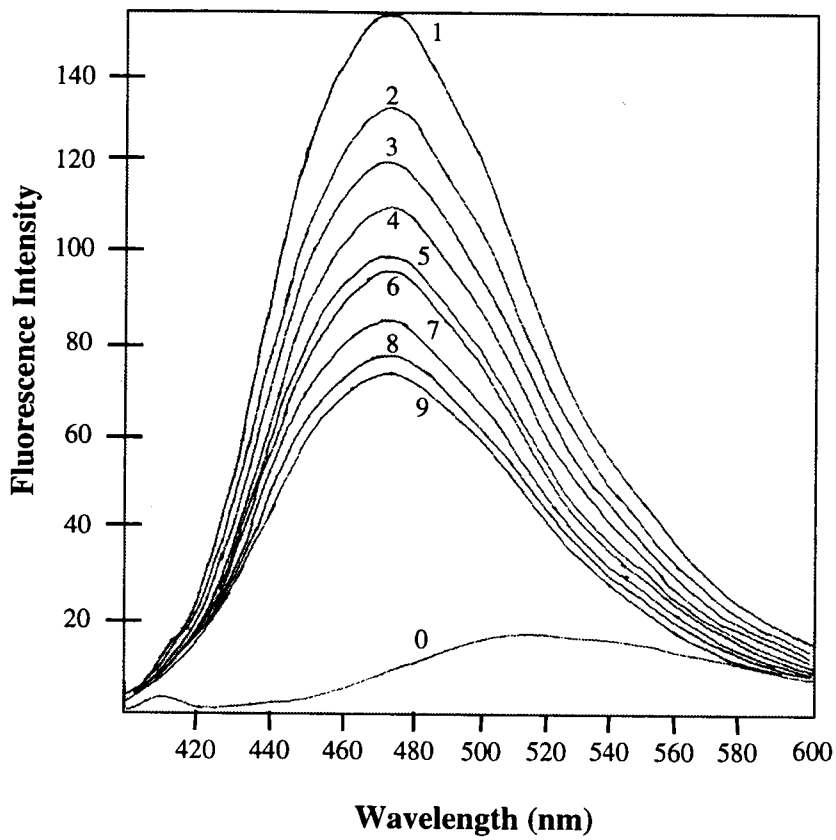


Fig. 1.5 : Effect of [α-ketoglutarate] on fluorescence intensity of ANS-labeled GABA-T

GABA-T (5 μM) was labeled with ANS (50 μM), incubated with increasing concentrations of α-ketoglutarate in 20 mM potassium buffer, pH 7.0, and fluorescence intensity covering 400-600 nm measured after excitation at 360 nm.

0 - free ANS
1 - ANS-labeled GABA-T in absence of α-ketoglutarate
2-9 - ANS-labeled GABA-T in increasing concnetrations (10-80 mM) of α-ketoglutarate

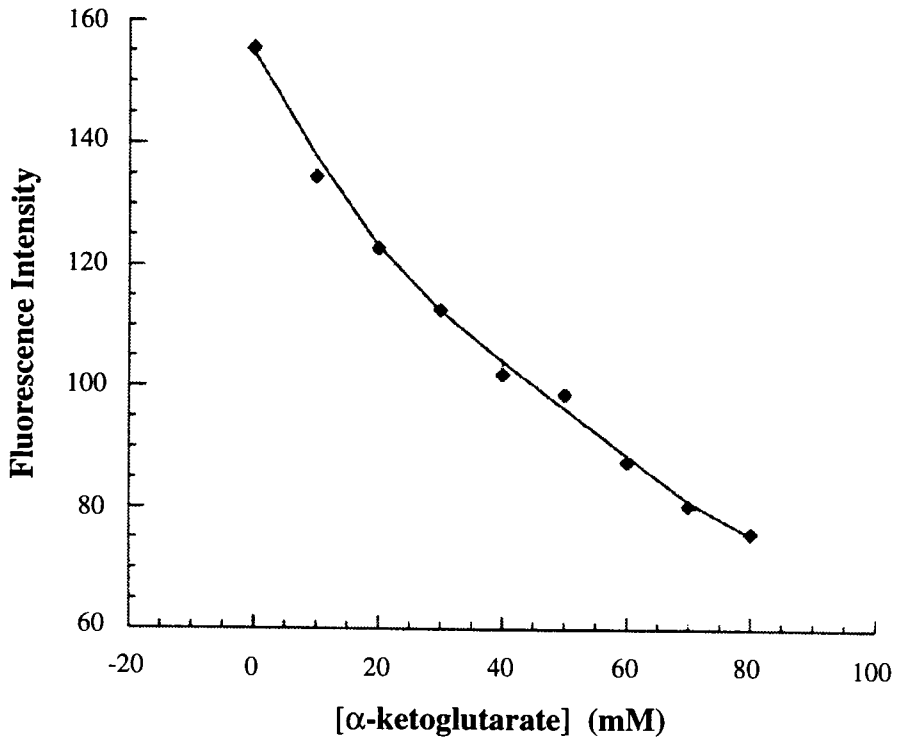


Fig. 1.6 : Fluorescence intensity of ANS-labeled pGABA-T versus [α-ketoglutarate]

4-aminobutyrate aminotransferase was labeled with ANS and the fluorescence intensity at 480 nm measured in the presence of increasing concentrations of α-ketoglutarate.

binding of the substrate, a decrease in fluorescence intensity of ANS was observed. This trend continued with increasing concentrations of α-ketoglutarate, indicating that the fluorescence was being quenched by the substrate. The activity of the labeled enzyme was also measured, and it was found to be inhibited by 26% in the presence of the label. Further studies carried out to determine the type of inhibition by keeping the concentrations of the substrates constant and varying [ANS] is shown in Fig. 1.7. The inhibition was non-competitive with an apparent K_I of 1.0×10^{-4} M^{-1}.

Stopped-flow Measurements

The characteristic ANS binding to hydrophobic sites on the protein was once again utilized to monitor the rate of dissociation of the dimeric enzyme at acid pH. The mixing of 20 mM potassium phosphate buffer (pH 7.0) and a 10 fold excess of 50 mM potassium phosphate (pH 4.2) resulted in a buffer with a pH of 5.0. Fig. 1.8 shows the results for the dissociation of the enzyme monitored by stopped-flow measurements of ANS-labeled enzyme. The dead time for the mixing of the buffer was 5 ms. When samples of ANS-enzyme at pH 7.0 were brought to pH 5.0 as described in the experimental procedures, an increase in the fluorescence intensity was observed immediately after the dead time of mixing. A relaxation time of 18 ms was shown for the dissociation process, but attempts to measure the rate constant associated with the formation of the dimeric species of the ANS-enzyme upon increasing the pH of the solution from 5.0 to 7.0 were unsuccessful because the decrease in fluorescence was too fast to be detected in the stopped-flow apparatus. Similar observations have been reported for the association of other PLP-dependent enzymes such as the β-subunit of tryptophan synthetase (Chaffotte and Goldberg, 1983) and aspartate aminotransferase (Leistler, *et al.*, 1992).

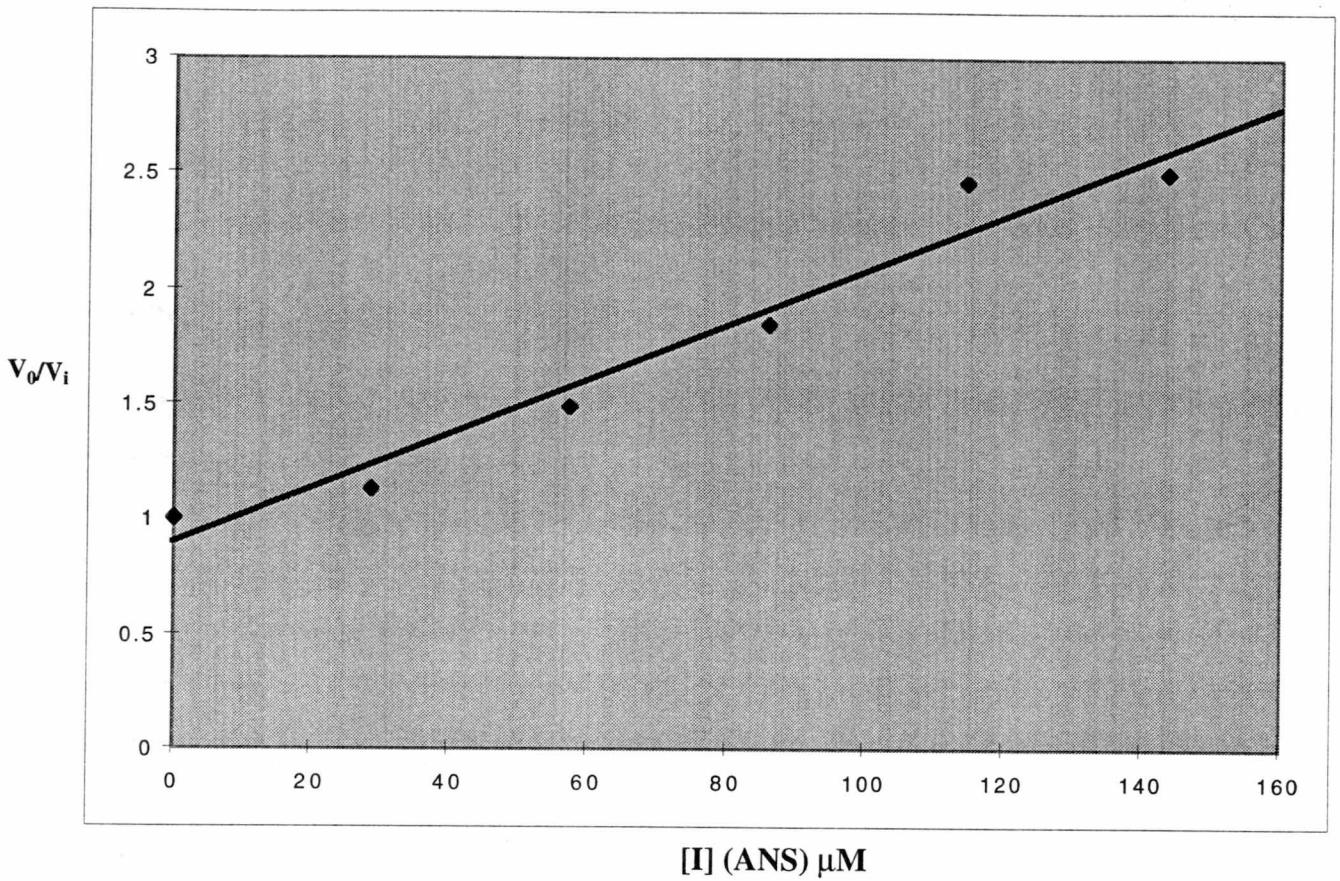


Fig. 1.7 : Plot of V_0/V_i vs. [I]

4-aminobutyrate aminotransferase (5μM) was labeled with increasing ANS concentrations. The velocity of the reaction was determined at constant substrate concentrations (10mM αKG and 20mM GABA) in the absence and presence of increasing [ANS]. The apparent K_I was determined by using the relationship: $V_0/V_i = 1+[I]/ K_I$ where a plot of V_0/V_i gives a slope = $1/K_I$

V_0 - reaction velocity in absence of inhibitor

V_i - reaction velocity in presence of inhibitor

[I] - inhibitor concentration

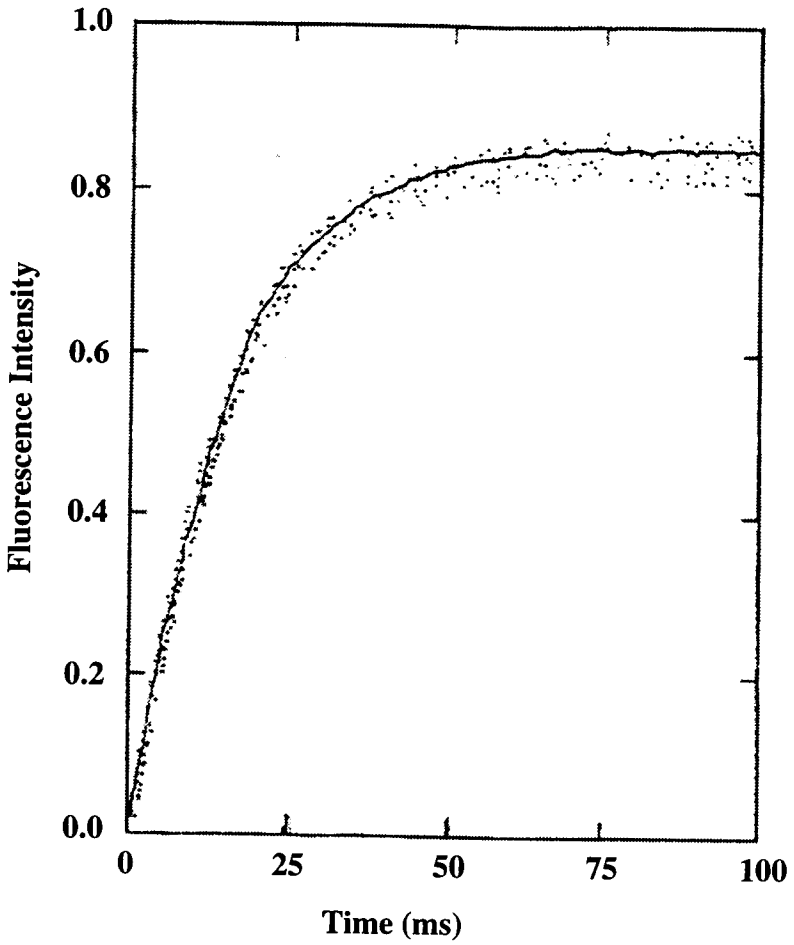


Fig. 1.8 : Dissociation of 4-aminobutyrate aminotransferase (kinetics)

Dissociation of 4-aminobutyrate aminotransferase at pH 5.0 monitored by increase in the fluorescence of bound ANS. A solution containing enzyme (50 μM) and ANS (400 μM) in 20 mM potassium phosphate (pH7.0) was diluted 10-fold with a solution of 50mM potassium phosphate, pH4.2, in the mixing chamber of the stopped-flow apparatus to a final pH of 5.0. The increase in fluorescence intensity was recorded immediately after mixing. The dead time was 5 ms.

The data from the various measurements seem to indicate that GABA-T adopts a specific stable structure at acid pH, and that this folded state is stabilized by well-defined tertiary contacts which are comparable to those found in the native enzyme. Buchner *et al.*, 1990, showed that at pH values below 3.0, a murine monoclonal antibody (MAK33) acquires a stable conformation that is different from either the native or the denatured form. This alternative folded state exhibited certain characteristics of the molten globule state, but it differed from a molten globule in stability. The folded intermediate of GABA-T at pH 5.0 may resemble that of the alternative folded state of MAK33. Further studies using X-ray crystallography and NMR will throw more light on the structure of the enzyme. These techniques require relatively large amounts of pure protein, underscoring the need for utilizing biotechnology to obtain the human enzyme from non-tissue sources. Towards this aim subsequent chapters in this thesis describe the isolation of the human brain GABA-T cDNA and an attempt to express it using *E. coli* expression system.

Carboxypeptidase Y Digestion

The results obtained for enzyme activity after incubation with carboxypeptidase Y, are shown in Fig. 1.9. No loss of activity was observed after 3 h incubation of GABA-T in the presence of the protease. Phenylalanine, alanine and lysine were detected in the digest from thin layer chromatography (Fig. 1.10), while results from amino acid analysis indicated the presence of free phenylalanine, alanine, aspartic acid, lysine, leucine, isoleucine and serine following protease treatment. From the deduced amino acid sequences of the pig brain and liver enzymes (Kwon *et al.*, 1992; De Biase *et al.*, 1992), these amino acids correspond to the C-terminal amino acids. Since carboxypeptidase Y cleaves from the COOH-terminal, it could be inferred from the results that the loss of at least 7 amino acids from the C-terminal end of GABA-T does

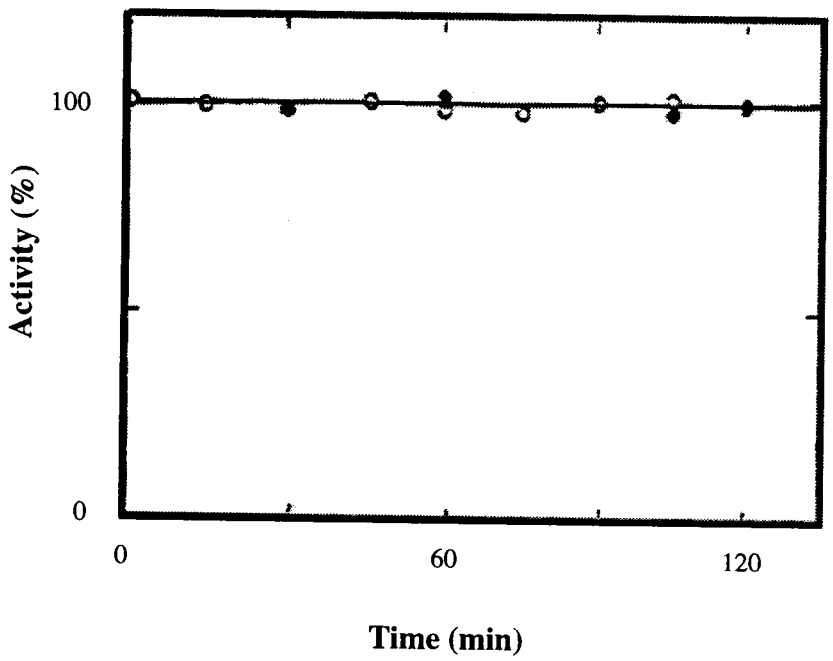


Fig. 1.9 : Effect of Carboxypeptidase Y digestion on the catalytic activity of 4-aminobutyrate aminotransferase.

The enzyme (1 mg/ml) was incubated in the abscence (o-o-o) and presence (•-•-•) of carboxypeptidase Y (20 μg/ml) at pH 7.6 at 25°C. Aliquots were removed from the incubation mixtures at different time intervals and tested for enzyme activity.

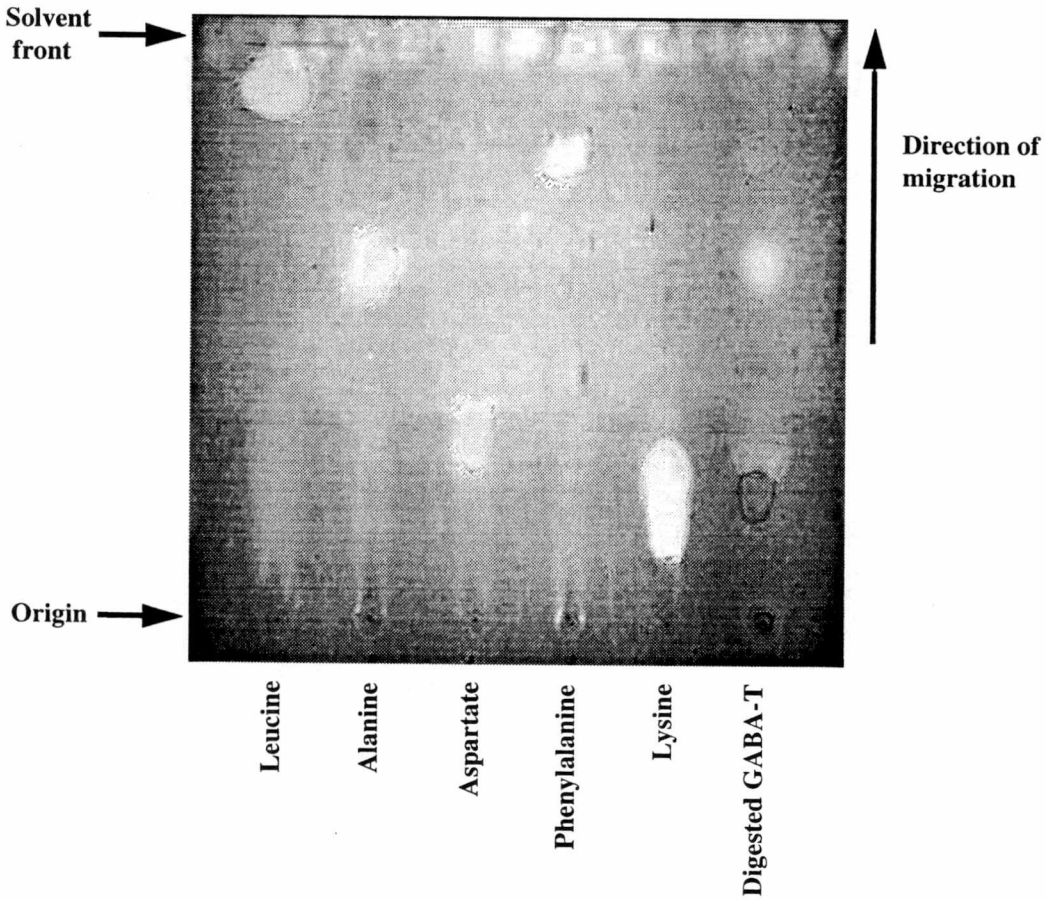


Fig. 1.10 : Thin layer chromatography of carboxypeptidase Y digested-GABA-T

An aliquot of the digest (10μL) after removal of the protein was applied on silica gel TLC plates together with amino acid standards. The samples were resolved using a mixture of n-butanol/pyridine/acetic acid/H_2O in the ratio 15:10:2:12 as solvent and the spots were visualized under UV light after spraying with 0.01% (w/v) fluorescamine in acetone.

not render the enzyme inactive, suggesting that proper folding prevents it from being susceptible to the protease.

Previous work by Kim and Churchich (1983) showed that the removal of 20 amino acids from the N-terminal of GABA-T by limited proteolysis using trypsin resulted in a truncated enzyme that was catalytically active. The results obtained with both proteases indicate that the N- and C-terminal residues are not required for activity and that their removal does not effect disociation to the inactive monomeric form.

CHAPTER 2

ISOLATION, SEQUENCING AND TISSUE DISTRIBUTION OF A cDNA ENCODING THE HUMAN BRAIN 4-AMINOBUTYRATE AMINOTRANSFERASE

INTRODUCTION

4-Aminobutyrate aminotransferase (GABA-T) is the enzyme that catalyzes the catabolism of the major inhibitory neurotransmitter, 4-aminobutyrate (GABA). GABA synthesis stems predominantly from glutamate in the brain in an irreversible reaction catalyzed by glutamic acid dehydrogenase(GAD). The concentration of GABA in the brain is therefore controlled by the action of GAD and GABA-T, both of which utilize pyridoxal-5'-phosphate as the coenzyme. GABA-T has been purified from various mammalian tissues including pig brain, (Churchich & Moses, 1981), human brain, (Cash et al., 1974; White & Sato, 1978), rat brain and liver (Bloch-Tardy *et al.*, 1971). The active enzyme was shown to be a homodimer with molecular weight of about 100 kDa.

The deficiency of GABA in the brain has been associated with certain neurological disorders such as epilepsy, since it has been shown that epileptic seizures could be terminated by direct injection of GABA into the brain of experimental animals (Hayashi, 1959). Overactivity of GABA-T causes seizures which can be alleviated by inhibitors of the enzyme. Several mechanism-based inhibitors of GABA-T have been designed and studied, although some of them are not very selective for GABA-T and affect the action of other PLP requiring enzymes. 4-aminohex-5-enoate, (Vigabatrin),

has been shown to be selective for GABA-T and is currently being used as a drug in the management of epilepsy.

Most of the studies on GABA-T have been carried out using other mammalian enzymes rather than the human form because of the difficulty in obtaining fresh human tissue. However, in order to test compounds being proposed for the treatment of diseases involving GABA-T in humans, it will be necessary to use the human enzyme.

As a first step towards the expression of the enzyme using current methods in biotechnology, the cDNA of human GABA-T has been isolated from a human brain cDNA library and sequenced. Its corresponding amino acid sequence was deduced for comparison with the known sequences from other mammalian and non-mammalian sources. Subsequent expression will hopefully provide a readily available source of the enzyme to facilitate thorough studies, including the utilization of mutagenesis to produce mutant forms of the enzyme to help in the design of novel drugs for the treatment of epilepsy.

EXPERIMENTAL PROCEDURES

Materials

A human brain cDNA library (frontal cortex, 85 yr. old female), in λZAP®II was purchased from Stratagene, La Jolla California. Salmon sperm DNA (type II, sodium salt) bovine serum albumin (fraction V), polyvinyl pyrrolidone and all antibiotics were obtained from Sigma Chemical Company, St Louis, Missouri. Ficoll (type 400) was purchased from Pharmacia. Biotrans nylon membranes and

[α-^{32}P]dCTP were purchased from ICN Biochemicals, Inc., Costa Mesa, California, while [α-^{35}S]dATP was obtained from Amersham Corporation, Arlington Heights, Illinois. Agarose and low melting agarose were purchased from FMC Bioproducts, Rockland, Maine and Bethesda Research Laboratories, Inc., Gaithersburg, Maryland, respectively. Restriction enzymes were from either New England Biolabs, Inc., Beverly, Massachusettes, or Stratagene, La Jolla, California, and the Prime-a-Gene labeling system was from Promega Corporation, Madison, Wisconsin. The Sequenase Version 2.0 Sequencing kit was purchased from United States Biochemical Corporation, Cleveland, Ohio. Synthetic oligonucleotides were purchased from Oligos, Etc.,Wilsonville, Oregon. Human multiple tissue Northern blot and ExpressHyb hybridization solution were purchased from Clontech Laboratories, Palo Alto, California.

Preparation of radiolabeled probe for screening

A fragment of the cDNA for pig brain GABA-T cloned in pET11d (Park, *et al.*, 1993), was obtained by double digestion of the recombinant DNA with K*pn* I and S*ma* I. Neither of the restriction enzymes cut the vector, and the cDNA fragment contained the cofactor binding site, which would be expected to have high sequence homology among species. A reaction mixture was set up using 80 μg recombinant DNA in a total volume of 90 μl sterile ddH2O, followed by addition of 10 μl of 10X Universal buffer (1 M potassium acetate, 250 mM Tris-acetate buffer pH 7.6, 100 mM magnesium acetate, 5 mM β-mercaptoethanol, 100 mg/ml bovine serum albumin), 50 units K*pn* I and 48 units of S*ma* I. The mixture was initially incubated at 25°C for 1 h and subsequently transferred to 37°C waterbath and incubated for another 2 h. The reaction was stopped by heating the mixture at 65°C for 20 min., cooled to room temperature, and 25 μl of 6X DNA loading buffer (0.25% bromophenol blue, 40% w/v

sucrose in water) were added, mixed thoroughly and applied on a preparative gel (1% low melting agarose) together with DNA molecular weight standards and undigested recombinant DNA. Electrophoresis was carried out at 4°C for 3 h at 25 mAmp using 1X Tris-acetate EDTA [0.04 M Tris-acetate, pH 8.0, 1 mM EDTA, (TAE)] buffer as the running buffer. The gel was stained in 0.01% ethidium bromide for 20 min, rinsed in distilled water for 10 min and viewed under UV light. The K*pn* I/S*ma* I digested fragment corresponding to approximately 670 base pairs (bp), was excised and the DNA fragment extracted from the gel by following the phenol-extraction method described in Sambrook *et. al.* (1985). Three volumes of 10 mM Tris-HCl buffer,containing 1 mM EDTA (TE buffer), pH 8.0 were added and heated at 65°C until the agarose melted completely. The mixture was vortexed briefly and allowed to cool to room temperature. An equal volume of phenol equilibrated in TE buffer was added, vortexed and spun in the microfuge at top speed for 5 min at room temperature. The top aqueous layer was carefully pipetted into a clean microfuge tube and the phenol extraction repeated. The aqueous layer was removed into a clean tube and extracted again with an equal volume of a mixture of phenol : chloroform : isoamyl alcohol (25 : 24 : 1), and a final extraction was carried out using chloroform : isoamyl alcohol (24 : 1). The aqueous layer was pipetted into a clean eppendorf tube and 1/10 volume of 3 M sodium acetate, pH 5.2, and 2.5 times the volume of ice-cold absolute ethanol added, mixed thoroughly and left at -20°C overnight. The mixture was centrifuged at 4°C at top speed in a microfuge to pellet the DNA, and the DNA was rinsed by addition of 70% ethanol and re-centrifuged to pellet the DNA. Traces of ethanol were removed by vacuum drying, and the DNA was dissolved in TE before labeling.

Radiolabeling of DNA fragment

Radiolabeling of the DNA was carried out by the random-primer labeling method using the protocol supplied with the Prime-a-Gene labeling kit from Promega. Approximately 25 ng of the K*pn* I/S*ma* I DNA fragment (5 µl), was denatured by heating at 95-100°C for 2 min and quickly chilled on ice. The reaction mixture was set up in a microfuge tube on ice by addition of the components in the order stated : Sterile nuclease-free water to give a final reaction volume of 50 µl was pipetted into an eppendorf tube and 10 µl 5X labeling buffer, 2 µl of a mixture of dNTPs (prepared by mixing 1 µl each of dATP , dTTP , dGTP giving a final concentration of 20 mM each), 5 µl denatured DNA, 2 µl nuclease-free BSA (400 µg/ml), 5 µl [α-^{32}P]dCTP (50 µCi, 3000 Ci/mmole) and 1 µl Klenow fragment (5 units). The components were mixed gently, incubated at room temperature for 5 h and reaction terminated by heating at 95-100°C for 2 min and quickly chilling on ice. EDTA was added to a final concentration of 20 mM, and the labeled DNA was purified on a Sephadex G-50 column equilibrated with TE buffer pH 8.0. The level of radioactivity of the labeled DNA was 0.78 x 10^5 cpm/µl. The radiolabeled DNA was stored at -20°C until needed.

Screening of the library

The human brain cDNA library in the λZAP®II vector was screened using the radiolabeled DNA probe. The library was serially diluted and aliquots were plated with 200 µl XL1- Blue cells (OD_{600} = 0.5) in 3.0 ml of top agar/plate on 100 mm LB agar plates, followed by incubation at 37°C for 8 h. The number of plaques/plate was counted and used to determine the titer of the stock. Based on this, the stock was diluted and approximately 50,000 pfu/plate was plated with 600 µl of host XL1-Blue cells (OD_{600} = 0.5) in 6.5 ml of top agar/plate on 150 mm LB agar plates. The plates were incubated at 37°C for about 8 h until plaques appeared and then transferred to

4°C. For each plate, duplicate filter replicas of the plaques were lifted onto Biotrans nylon membranes. The first filter was placed for 2 min while the duplicate filter was placed for 5 min on the plates. After lifting the plaques, the filters were treated by floating them in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 seconds, immersing them for an additional 60 sec and quickly transferring them into neutralization solution (1.5 M NaCl in 0.5 M Tris-HCl, pH 8.0) for 5 min. The filters were rinsed in 3X standard saline citrate (SSC) for 30 sec and placed on Whatman 3 mm filter to air dry. The phage DNA was immobilized on the membrane by baking at 80°C for 3 h.

Pre-hybridization was carried out in a sealed plastic bag using a 50% formamide solution [50% formamide, 6X SSPE (20x SSPE contains 175.3 g NaCl, 27.6 g NaH_2PO_4. H_2O and 7.4 g of EDTA in a total volume of 1 l H_2O with pH adjusted to 7.4 with 10 N NaOH), 5X Denhardt's solution, 0.5% SDS and denatured sonicated salmon sperm DNA (100 μg/ml)], 2-4 ml of hybridization solution per 100 cm^2 of filter, for 5 h at 42°C inside a rotating hybridization chamber. Hybridization was performed overnight under identical conditions using a heat-denatured radiolabeled DNA fragment probe at a radioactivity level of $0.5x10^6$ cpm/ml. Two post-hybridization washes at room temperature for 30 min each with several changes of wash solution, 2X SSC/0.05% SDS were followed by two 60°C washes for 30 min each in 2X SSC/0.1% SDS. The filters were blotted on Whatman 3 mm paper, covered with Saran wrap and exposed at different lengths of time at -70°C on Kodak X-OMAT X-ray film (Sigma) in cassette with intensifying screen.

The positive clones identified in the primary screening were picked from the agar plates into sterile SM buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 8mM

$MgSO_4$, 0.01% gelatin), vortexed and kept at room temperature for 1 h to release the plaques. For secondary screening, dilutions of the plaques in the SM buffer giving about 100-1000 pfu/plate were added to 200 µl XL1-Blue cells (OD_{600} = 0.5) in 3 ml top agarose, plated on LB/agar plates and incubated at 37°C for the plaques to grow. The plaques were lifted onto Biotrans nylon membranes, immobilized on the membranes by baking at 80°C and used in hybridization reactions with the radiolabeled fragment, as previously described, to locate the positive plaques. The process described above was repeated for the tertiary screening using the diluted plaques from the secondary screening. Three positive clones picked after this step were used in *in vivo* excision reactions to obtain the pBluescript phagemid with the GABA-T cDNA insert from the λZAP® as decribed below.

In vivo excision of pBluescript phagemid with insert from λ ZAP® II vector

The excision protocol using Ex Assist/SOLR system described by Stratagene for λZAP®II vector was followed. In a 50 ml conical flask, 200 µl of XL1 - Blue cells (OD_{600} = 1.0), 100µl of the phage stock in SM containing >1 x 10^5 phage particles and 1 µl of ExAssist helper phage were mixed and incubated at 37°C for 15 min. After incubation, 3 ml of sterile 2XYT media (10 g NaCl, 10 g yeast extract, 16 g bacto-tryptone per liter of distilled water) were added and re-incubated at 37°C with constant shaking for 5 h. The flask and contents were heated at 70°C for 20 min in a water bath, cooled and spun at 4,000 x g for 15 min. The supernatant fraction which contained the rescued phagemid with the insert, was decanted, aliquoted and stored at 4°C. An aliquot (1 µl) of this stock was added to 200 µl of SOLR cells (OD_{600} = 1.0) and incubated at 37°C for 15 min, after which aliquots were plated on LB agar plates containing 50 µg/ml ampicillin (LB/AMP) and incubated at 37°C overnight. Colonies

appearing on the plates were grown in LB/AMP media and glycerol stocks were prepared and stored at - 80°C.

Preparation of pBluescript phagemid with the insert GABA-T cDNA and sequencing

The method for preparation of plasmid DNA described by Sambrook *et. al.*, (1989), was followed. All the solutions used were either autoclaved or filter-sterilized as appropriate. A seed culture of SOLR cells containing the recombinant phagemid was transferred into 250 ml LB/AMP medium and incubated at 37°C overnight with constant shaking. The cells were harvested by centrifugation at 4,000 rpm for 15 min in a Sorvall GS 3 rotor. The cells were re-suspended in 25 ml ice-cold STE buffer (0.1 M NaCl, 10 mM Tris HCl, pH 8.0, 1 mM EDTA, pH 8.0), and re-centrifuged as above. The rinsed cells were re-suspended in 10 ml solution I (50 mM glucose, 25 mM Tris HCl, 10 mM EDTA), and 1 ml of freshly prepared lysozyme (10 mg/ml) and 20 ml of freshly prepared solution II (0.2 M NaOH and 1%SDS) were added. The centrifuge tube was inverted gently and left at room temperature for 10 min. After the incubation, 15 ml of ice-cold solution III (3 M potassium and 5M acetate, prepared by addition of 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml sterile distilled water) were added. The tube was inverted several times, kept on ice for 10 min and centrifuged at 4,000 rpm for 15 min at 4°C in Sorval GS 3 rotor. The supernatant was filtered through 4 layers of sterile cheese cloth into a 250 ml centrifuge bottle and 0.6 volume of isopropanol was added, mixed well and allowed to stand at room temperature for 10 min. The nucleic acids were recovered by centrifuging at 5,000 rpm for 15 min at room temperature and the supernatant discarded. The tube was inverted to allow the last drops of the supernatant to drain and the pellet rinsed with 70% ethanol at room temperature.

The pellet was dissolved in 3 ml TE buffer and treated as follows to remove the contaminating mRNA. The solution was transferred into 15 ml autoclaved Corex tube and 3 ml ice-cold sterile 5 M lithium chloride solution were added and mixed well. Following centrifugation at 10,000 rpm for 10 min at 4°C in a Sorvall SS34 rotor, the supernatant fraction was transferred into a 30 ml Corex tube, and an equal volume of isopropanol was added, mixed well and centrifuged as above. The supernatant fraction was decanted, and the pellet was rinsed with 70% alcohol at room temperature and subsequently re-dissolved in 250 µl TE containing DNase-free pancreatic RNase (20 µg/ml).

The mixture was incubated at room temperature for 30 min and 250 µl of 1.6 M sodium chloride solution containing 13% polyethylene glycol (w/v) were added, mixed well and centrifuged at 12,000 x g for 5 min in a microfuge. The supernatant was removed by aspiration, and the pellet was dissolved in 400 µl TE, pH 8.0. The DNA was then extracted by addition of phenol-chloroform and precipitated by addition of 100 µl filter-sterilized 10 M ammonium acetate and 2 volumes of ethanol and left at -80 °C for10 min. The plasmid DNA was recovered by centrifugation at 12,000 x g for 5 min at 4°C, rinsed with 70% alcohol, re-centrifuged to pellet the DNA and vacuum-dried to remove the traces of alcohol. The DNA was dissolved in 10mM TE, pH 8.0, and aliquots were taken for sequencing.

Sequence analyses were performed by the dideoxy chain termination method (Sanger *et al.*, 1977), using [α-^{35}S]dATP and the Sequenase Version 2.0 sequencing kit from USB and following the manufacturer's protocol for the sequencing reactions. The double-stranded plasmid DNA with the GABA-T cDNA insert and synthetic oligonucleotides were used as template and primers, respectively. The primer walking

method (Kieleczawa *et al.*, 1992) was followed and the sequencing products were resolved in 6% polyacrylamide/8 M urea gels by running the gel at constant power (60 W) with 1X Tris Borate EDTA buffer (89 mM Tris borate, 2 mM EDTA) as the running buffer. At the end of the run, the gel was fixed in 12% methanol/10% acetic acid for 20 min, transferred onto Whatman 3 mm paper, vaccum dried and exposed on X-ray film at room temperature. The sequence was read after the development of the film.

Nucleotide and Deduced Amino acid Sequence Analyses

The nucleotide sequence and the corresponding amino acid sequence were deduced with the help of the GCG computer program. DNA and protein sequence homology search were carried out using the Genbank data base.

Northern Blot Hybridization

pBluescript phagemid containing the human brain cDNA for GABA-T was digested with B*am* HI and the fragment separated by electophoresis on 0.8% low melting agarose. The fragment was excised and purified following the phenol extraction method described earlier. The purified fragment was random-prime labeled using the Prime-a-Gene Labeling System and [α-^{32}P]dCTP. The level of radioactivity of the labeled fragment was 0.77 x 10^6 cpm/μl.

Pre-hybridization and hybridization were carried out following the protocol outlined in Clontech protocol # PT 1190-1. The ExpressHyb solution was heated at 68°C, stirred gently to dissolve any precipitate and 5 ml of this solution pipetted into a plastic bag containing the multiple tissue Northern blot (Clontech). The bag was sealed after removing the air bubbles and incubated at 68°C with continuous shaking for 30

min. The labeled DNA probe (specific activity of 0.77 x 10^6 cpm/μl) was heated at 100°C in a boiling water bath for 5 min, quickly chilled on ice; 13 μl of this solution were added to 5 ml of fresh ExpressHyb solution at 68°C, and mixed thoroughly to give a final radioactivity level of 2 x 10^6 cpm/ml. The prehybridization solution was removed completely from the bag and replaced with the fresh hybridization solution containing the radiolabeled probe. Hybridization was carried out at 68°C for 1 h with continuous shaking. The blot was removed and washed in solution I (2 X SSC, 0.05% SDS) at room temperature for 40 min with continuous agitation and five changes of the solution. It was then transferred into solution II (0.1 X SSC, 0.1% SDS) incubated at 50°C in a water bath with constant shaking and washed for another 40 min with one change of fresh solution. The blot was removed, excess solution shaken off and immediately covered with plastic wrap and exposed at -80°C at different times to X-ray film in a cassette with double intensifying screen.

After the exposure, the probe was removed from the blot by boiling it for 10 min in distilled water containing 0.5% SDS and allowing it to cool in the water for another 10 min with frequent shaking. The blot was exposed for 72 h to check for the complete removal of the probe and the clean blot re-probed with radiolabeled β-actin (radioactivity level of 0.91 x 10^6 cpm/μl) at a final level of radioactivity of 0.5x10^6 cpm/ml to test for possible inter-lane variability in loading and transfer of the mRNA. The blot was washed as described earlier and exposed for different time intervals.

RESULTS AND DISCUSSION

Preparation of probe, Screening and Sequence Determination

Fig. 2.1, shows the results of the restriction digest of pET 11d with and without pig brain GABA-T cDNA insert. The size of the fragment obtained was approximately 670 bp which agreed with that deduced from the restriction map using the GCG program for the sequence of the pig brain cDNA (Kwon *et al.*, 1992). Out of six clones picked in the primary screening which had hybridized to the radiolabeled DNA probe, three of them were positive after the secondary and tertiary screening. Figs. 2.2A&B, show the results for the secondary and tertiary screening using the labeled fragment. These results showed that the pig brain cDNA had been used successfully to screen for the human brain cDNA, implying that there is some amount of homology between the two cDNAs. Fig. 2.3 shows the results from a segment of an autoradiogram of a sequencing gel. Each lane corresponds to one of the four dideoxynucleotide reactions. Fig. 2.4 shows the nucleotide sequence and the corresponding amino acid sequence predicted for the coding region using the GCG program. The open reading frame (ORF) consists of 1503 bp encoding a protein of 500 amino acids with a molecular weight of approximately 53 kDa and a predicted pI of 7.5. No poly(A) tail was observed at the 3' untranslated region, but a stop codon (TAA) was observed adjacent to the triplet codon for the C-terminal lysine, followed by a T+A-rich sequence. These observations agreed with the sequence deduced for the pig brain cDNA (Kwon *et al.*, 1992). In addition, the first 27 amino acids in the sequence for hGABA-T are rich in basic amino acids, arginine and histidine, and hydroxyl-containing amino acids, with no acidic residues. A similar observation had been made for pGABA-T (Kwon *et al.*, 1992), and rat brain GABA-T (Medina-Kauwe *et al.*, 1994) and had been assigned to the mitochondrial targeting sequence of the precursor GABA-T, as it fits the common

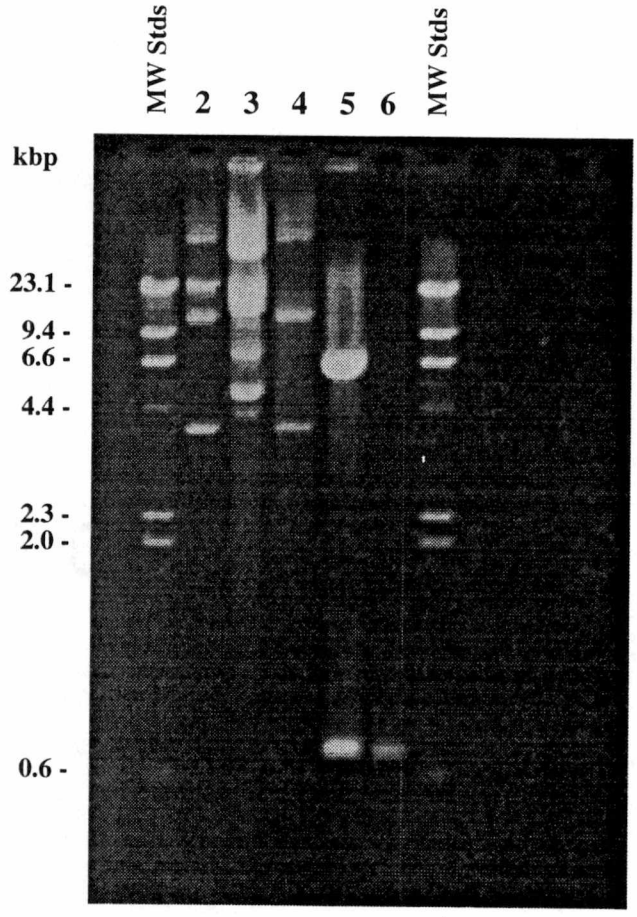
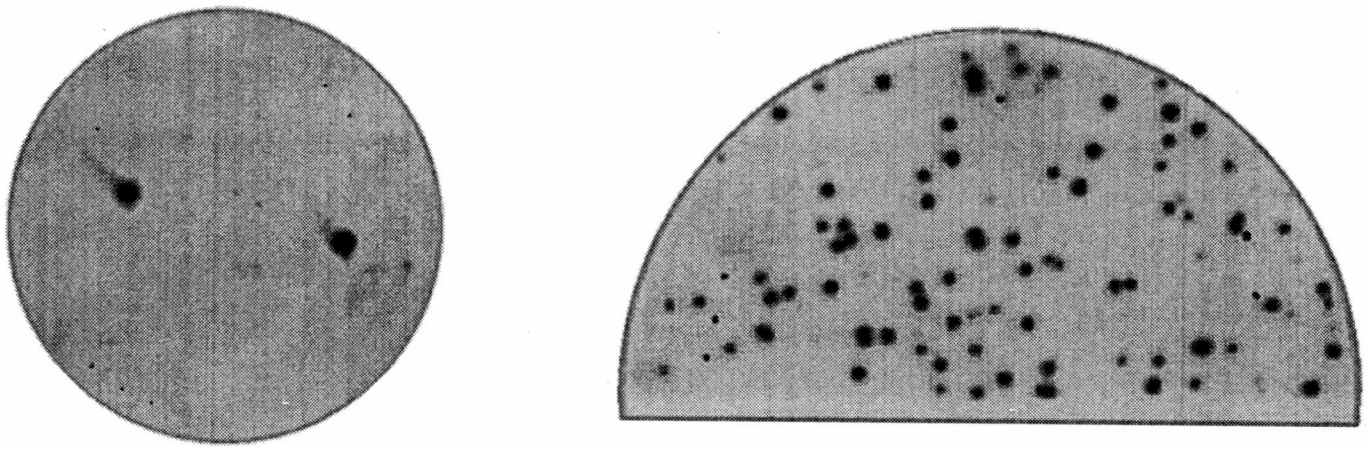


Fig. 2.1 : Restriction Enzyme Digestion of pET 11d + pig brain cDNA insert.

Electrophoresis was run on 1% agarose gel at 25 mAmps with 1X Tris-acetate EDTA (TAE) buffer as the running buffer.

Lane 2 - uncut pET 11d
3 - uncut pET 11d + pGABA-T cDNA
4 - digested pET 11d with K*pn*1/S*ma*1
5 - digested pET 11d-pGABA-T cDNA with K*pn*1/S*ma*1
6 - purified K*pn*1/S*ma*1 fragment from (5)



A. B.

Fig. 2.2 : Clones in λ-ZAP®II containing GABA-T cDNA after Secondary (A) and Tertiary (B) screening.

A fragment of 670bp for pig brain GABA-T cDNA was labeled and used to screen the human brain cDNA library in λ-ZAP®II vector (Stratagene) as described in the experimental procedures. A positive clone from the primary screening was isolated and amplified for the subsequent screening.

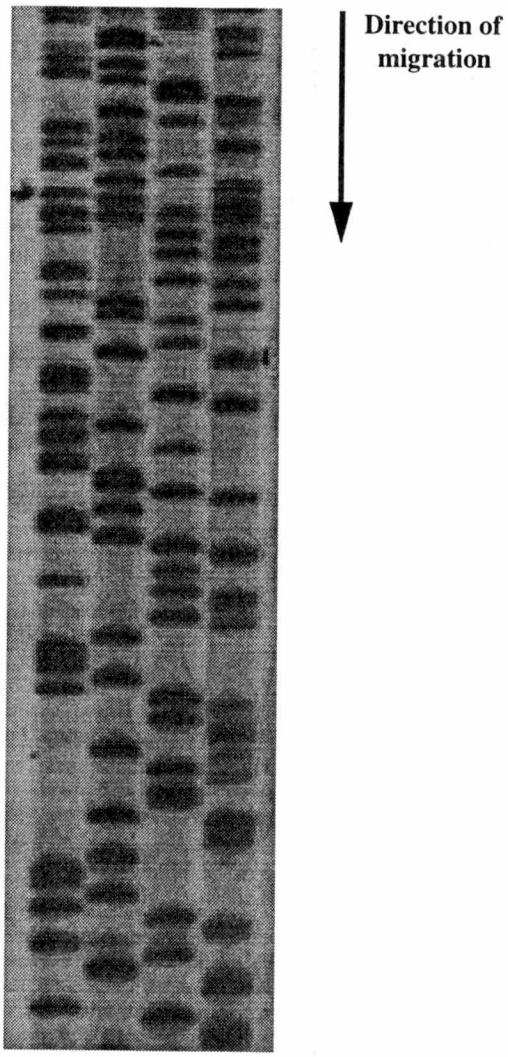


Fig. 2.3 : A segment of an autoradiagram of a sequencing gel.

Sequence analyses were performed by the Sanger dideoxy chain termination method (Sanger *et al.*, 1977) using [α^{35}S]dATP and the Sequenase Version 2.0 sequencing kit from USB and following the manufacturer's protocol for the sequencing reactions. The sequencing products were resolved in 6% polyacrylamide/8 M urea gels at constant power (60 W) with 1X Tris Borate EDTA buffer as the running buffer. The separated bands were visualized by autoradiography. Each lane corresponds to one of the four dideoxynucleotide reactions.

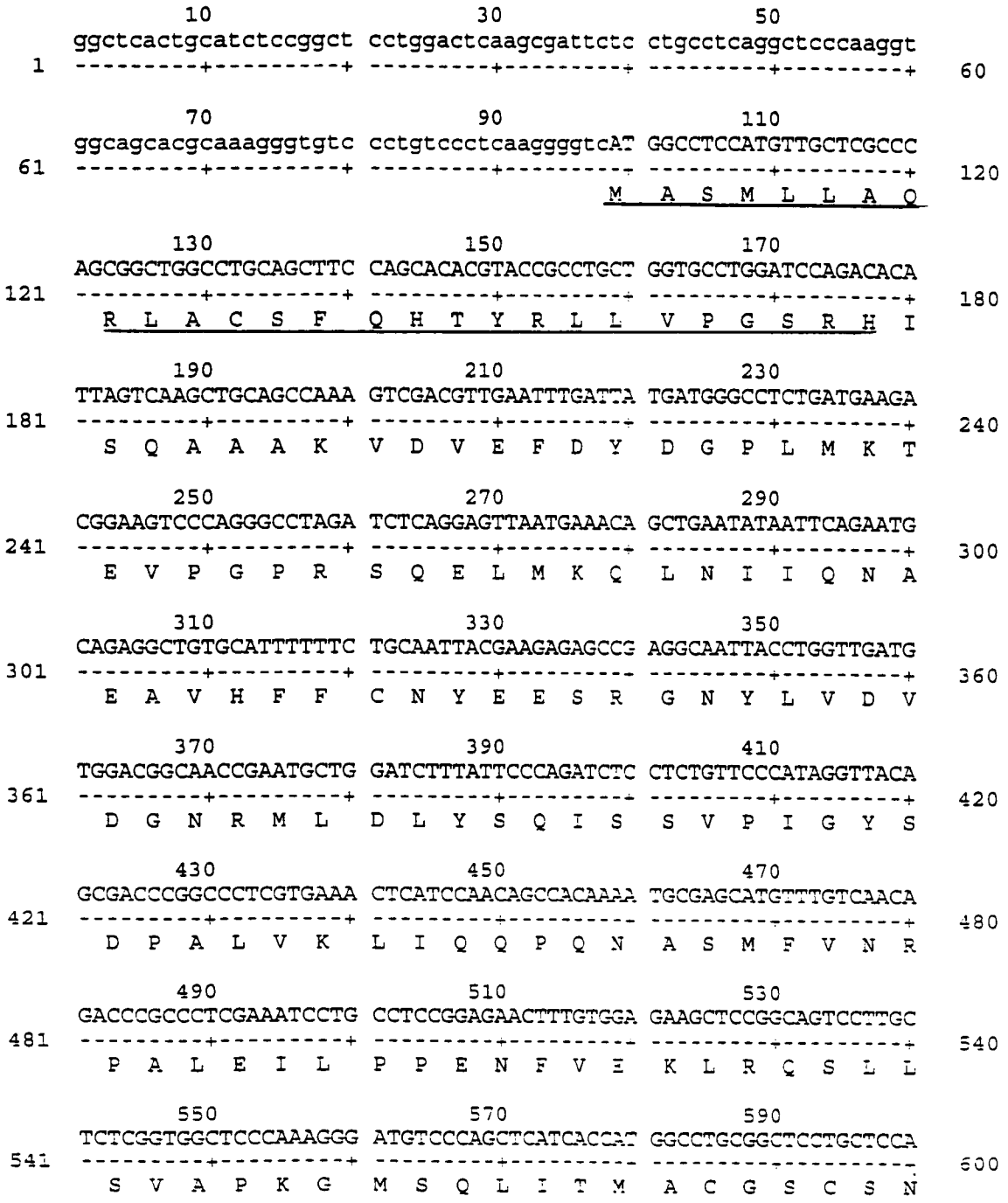


Fig. 2.4: **Nucleotide and predicted amino acid sequences of hGABA-T deduced using the GCG program.**

The 5'- and 3'-flanking regions are indicated (lower case). The first 27 amino acids (underlined) represent the presequence. The sequence has been deposited at the GenBank with accession number L32961.

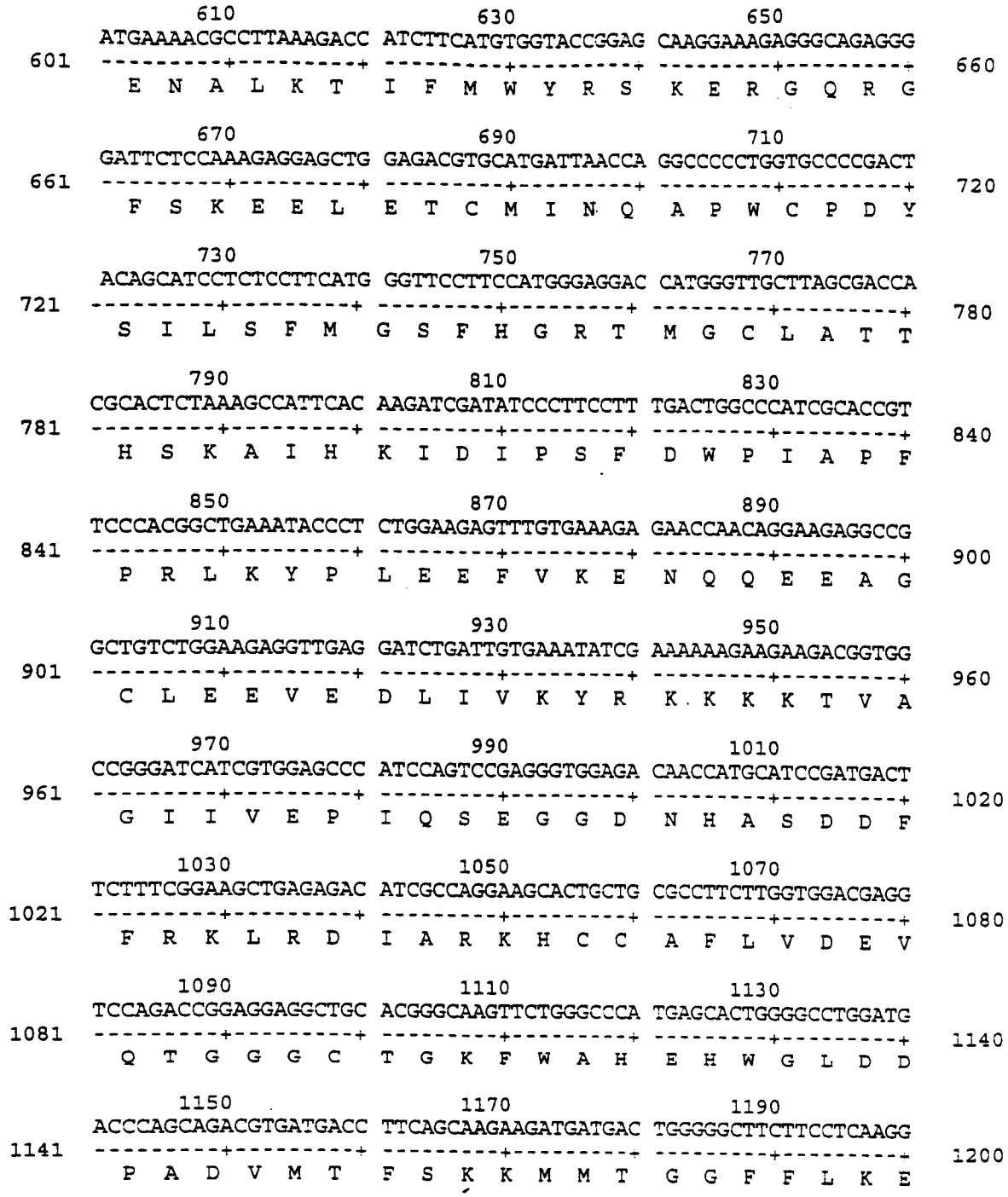


Fig. 2.4: Continued

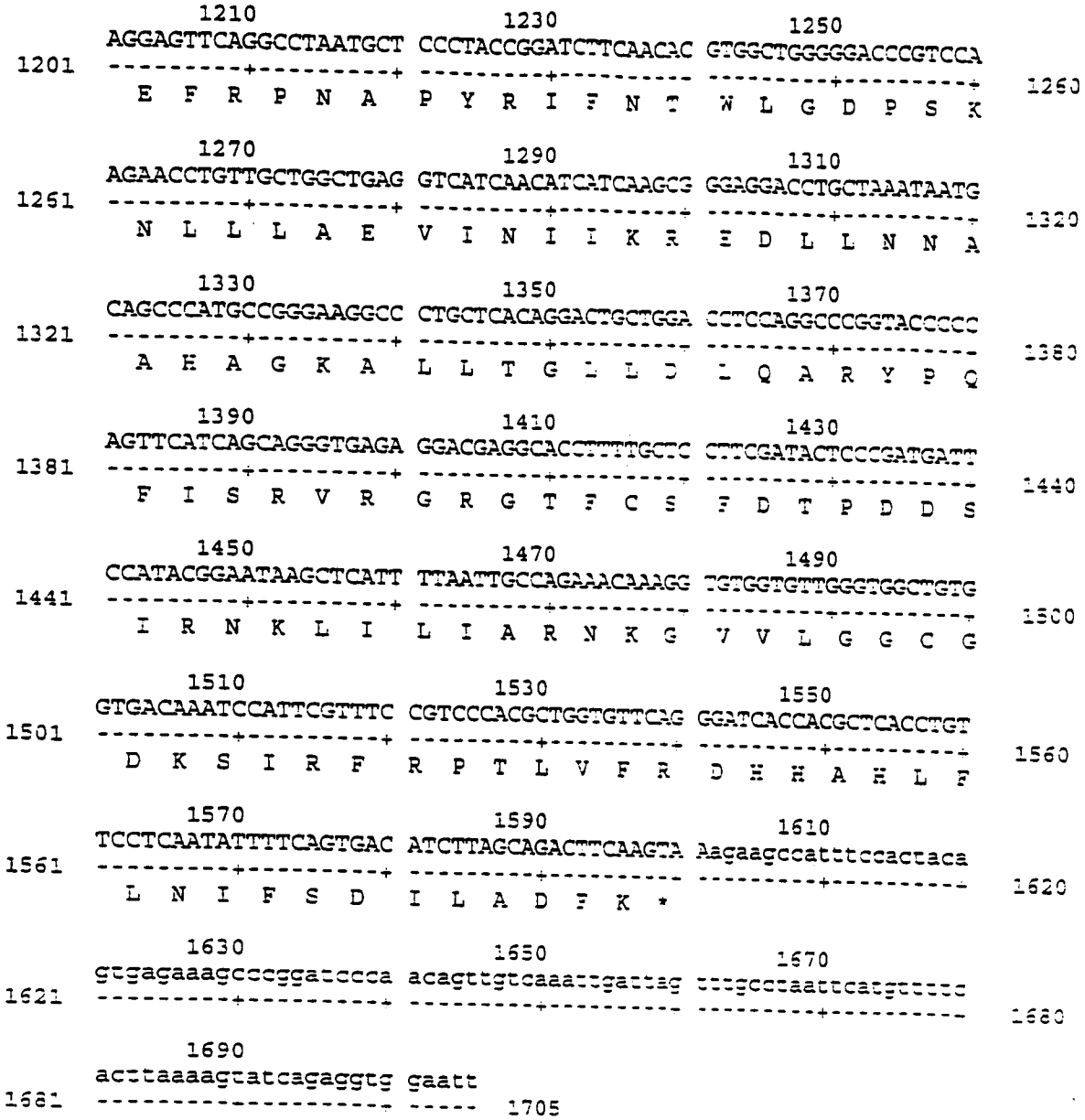


Fig. 2.4: Continued

description for sequences targeting mitochondrial enzymes (Reid, 1985; Roise *et al.*, 1985; von Heijne, 1986; Roise *et al.*, 1988; Roise and Schatz, 1988). All of these observations support the conclusion that the deduced sequence is that of the precursor form of the mitochondrial enzyme.

The predicted amino acid composition of the mature enzyme based on the cDNA sequence is shown in Table 2. There is an excess of positively-charged amino acids. The alignment of the amino acid sequence for the pig brain enzyme and that of the human enzyme from searches of the GenBank are shown in Fig. 2.5. It was also revealed that human brain GABA-T (hGABA-T) shared 95.4% sequence identity with pGABA-T, (Kwon *et. al.*, 1992) and pig liver GABA-T (De Biase *et al.*, 1992) and 94% with rat brain GABA-T (Medina-Kauwe *et al.*, 1994). A more recent report by De Biase *et al.*, (1995), in which the sequence of human liver GABA-T deduced largely by automated Edman degradation of proteolytic fragments revealed 96% identity to the pig enzyme. Almost 100% identity is observed for the human brain and liver enzymes. These observations revealed that GABA-T is highly conserved among the mammalian species. A lesser extent of sequence identity was observed among hGABA-T and the same enzyme from *A. nidulans* (Richardson *et al.*, 1989) 48%, *S. cerevisiae* (André & Jauniaux, 1990) 42%, and *E. coli* (Bartsch *et al.*, 1990) 28%.

Northern Blot Hybridization and Tissue Distribution of GABA-T cDNA

Results obtained for Bam HI digestion of pBluescript containing the insert hGABA-T cDNA are shown in Fig. 2.6. A fragment ~1.47 kbp was obtained and this agreed with the size of the fragment deduced from the restriction map of the cDNA (Appendix A2.1). Results from the Northern blot analysis, Fig. 2.7, panels B&C, indicated that the following tissues: heart, brain, liver, placenta, kidney and pancreas

Table 2. Amino Acid Composition of Mature 4-Aminobutyrate Aminotransferase

Amino acid	Number	Percentage of total (%)
Alanine	32	6.8
Asparagine	22	4.7
Aspartic acid	29	6.1
Arginine	26	5.5
Cysteine	12	2.3
Glutamic acid	30	6.3
Glutamine	18	3.8
Glycine	31	6.6
Histidine	12	2.3
Isoleucine	32	6.8
Leucine	44	9.3
Lysine	32	6.8
Methionine	13	2.7
Phenylalanine	29	6.1
Proline	26	5.5
Serine	28	5.9
Threonine	17	3.6
Tryptophan	5	1.1
Tyrosine	11	2.3
Valine	24	5.1
TOTAL	473	100

The analysis was done on the sequence in Fig. 2.3 and the predicted molecular weight was about 53 kDa.

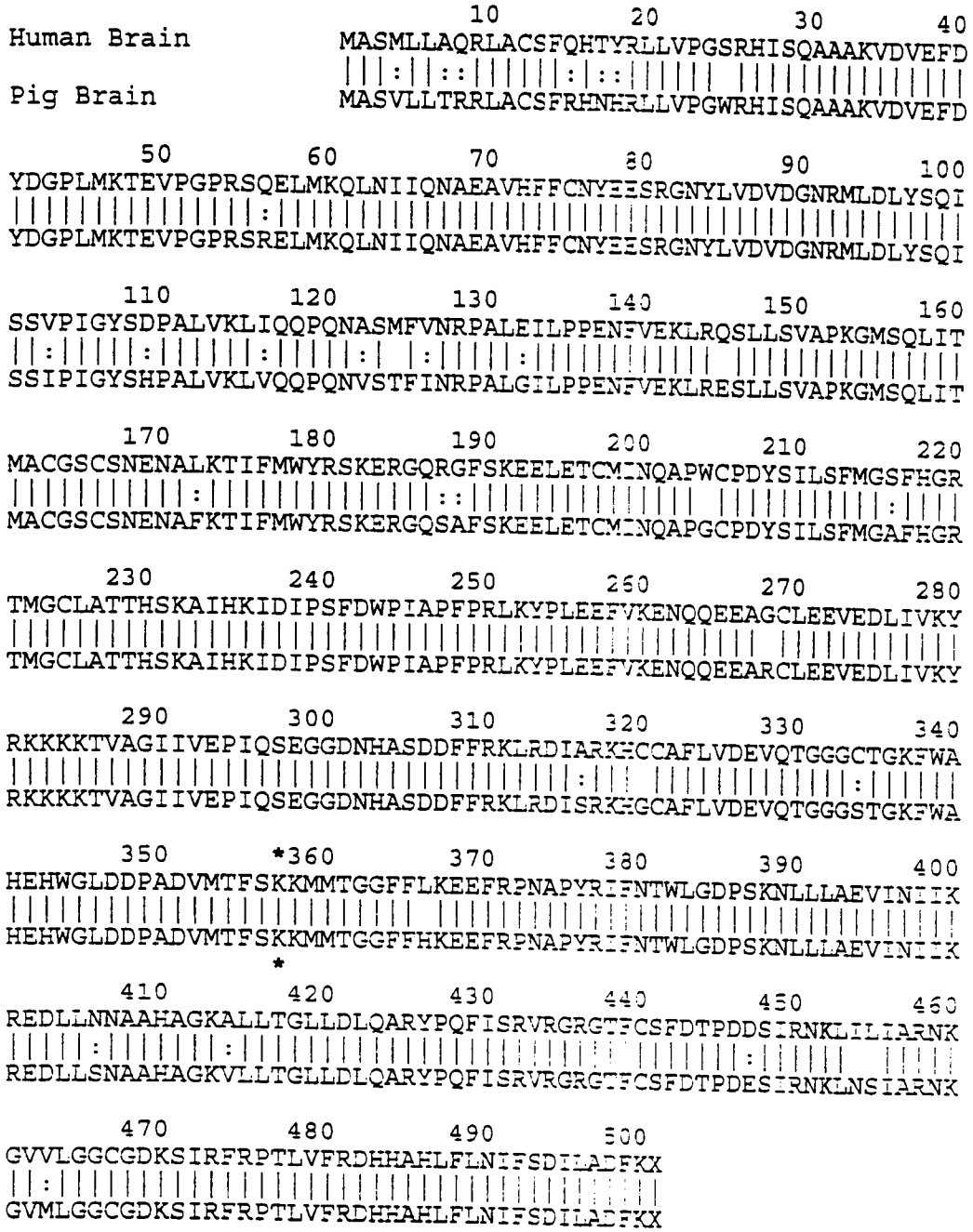


Fig. 2.5: Comparison of the deduced amino acid sequence of hGABA-T with the pig brain enzyme.

The GCG program was used to search the GenBank and compare the sequences. Vertical lines indicate identical amino acids, double dots, conservative substitutions and blank spaces, no homology. The cofactor binding Lys is marked by asterisk and the end of the sequence is denoted as X.

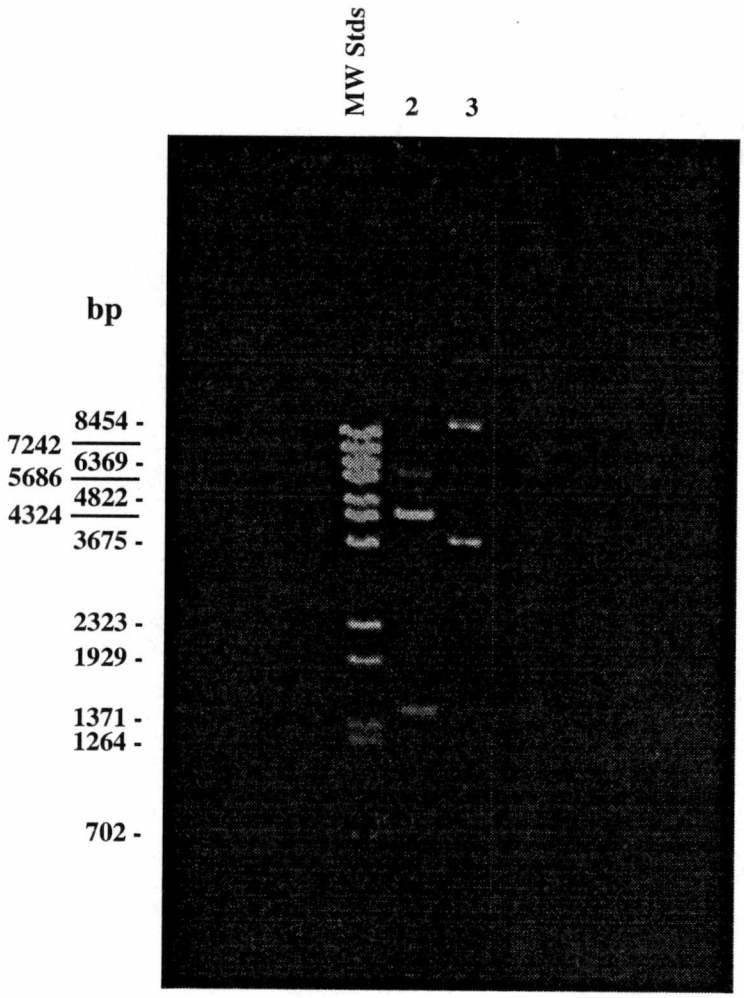


Fig. 2.6 : B*am* HI digestion of pBluescript containing hGABA-T cDNA

Lane 2 - digested recombinant plasmid DNA
Lane 3 - undigested recombinant plasmid DNA

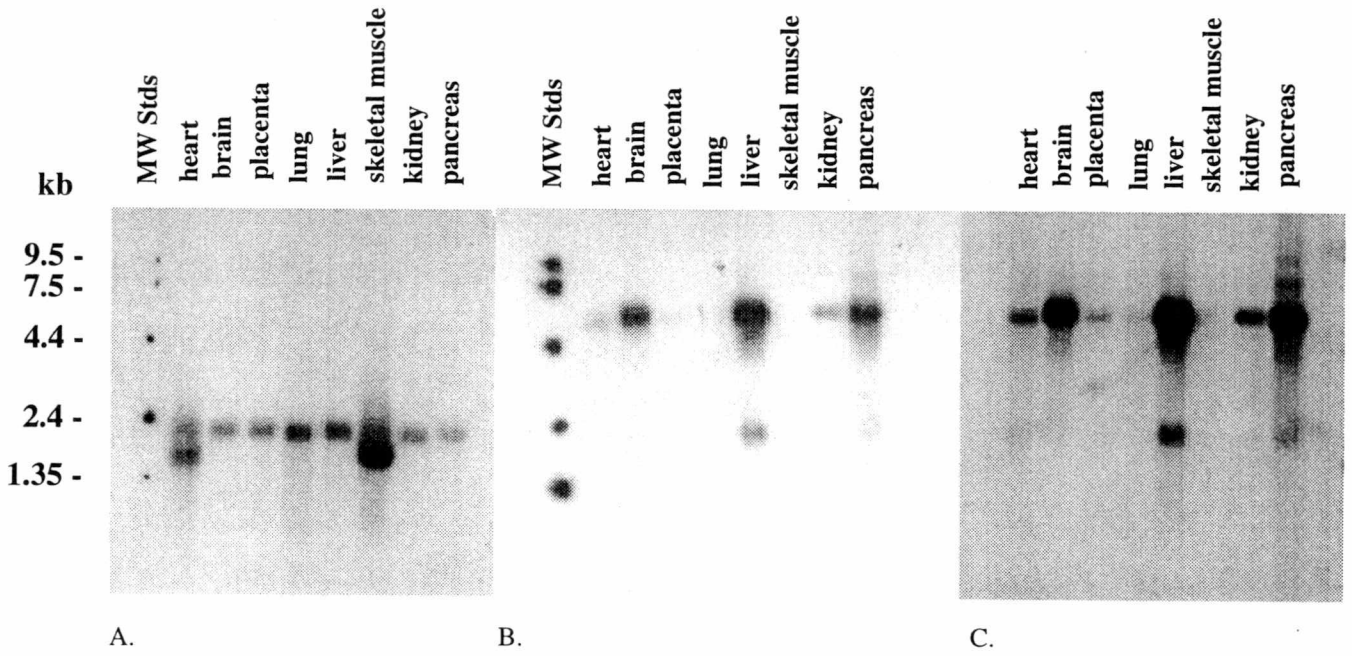


Fig. 2.7 : Tissue distribution of hGABA-T cDNA by Northern Blot analysis.

Multiple Tissue Northern Blot analysis of poly(A) - rich RNA from various human tissues (Clontech) hybridized to ^{32}PdCTP labeled ß-actin (A) and GABA-T cDNA (B and C). Exposure time for the ß-actin probed blot was one hour and 12 and 45 hours for blots B and C respectively.

possess the transcripts for GABA-T while the lungs and skeletal muscle did not. The major transcript in all the tissues corresponded to approximately 5.7 kb in size with the liver having the most intense band. The brain, liver and pancreas showed bands as early as 4 h after exposure and very intense bands after 12 h exposure (panel B) while the heart and kidney showed very faint bands with the lung and skeletal muscle showing no bands at all. Upon longer exposure, 45 h (panel C), three bands with approximate sizes; 2.1, 5.0 and 5.7 kb were observed for the liver while the pancreas showed five bands; 2.1, 5.0, 5.7, 7.5 and 9.0 kb. A fainter band, 2.1 kb was also observed for the brain and the heart in addition to the 5.7 kb band after this exposure period.

Probing with β-actin (Fig. 2.7, panel A), showed bands of about the same intensity of 2.0 kb for all the lanes with the heart and skeletal muscle having another band of 1.8 kb. These observations with β-actin confirmed that there were no inter-lane variabilities in the loading of the mRNA. The presence of two bands at 1.8 and 2.0 kb for the heart and skeletal muscle also agrees with the observation for the two tissues which are known to have two transcripts for β-actin.

The overall results from probing with GABA-T cDNA showed that the DNA is expressed by the heart, brain, placenta, liver, kidney and the pancreas, with the major transcript being 5.7 kb in size. Previous work by several workers has shown that GABA-T is present in several peripheral tissues such as liver, kidney, pancreas, reproductive organs and blood platelets in addition to the brain from rat, rabbit and mouse (Roberts & Bregoff, 1953; Nikolaeva & Vasil'ev, 1972; Vasil'ev *et al.*, 1973, Buzenet *et al.*, 1978; White and Sato, 1978; Wu *et al.*, 1978; White, 1979; Taniguchi *et al.*, 1979; Medina-Kauwe *et al.*,1994). Very recently GABA-T has been found in the heart and placenta of human (De Biase *et al.*, 1995). The results also indicate that

multiple transcripts are present in some of the tissues, e.g. the liver had three while the pancreas had five different transcripts. Such phenomena suggest the possibility of isozymes which had been reported earlier by some researchers, for mouse and rat brain, (Waksman & Bloch, 1968) and for guinea pig brain (Ho, *et al.*, 1975).

The overall results showed that the detection levels of GABA-T mRNAs are the following (in decreasing order): liver > pancreas > brain > kidney > heart > placenta, while transcripts were not detected in the lung and skeletal muscle even after 45 h exposure. These results agree with those observed recently by De Biase *et al.* (1995) and Medina-Kauwe *et al.* (1994), who detected the same using human liver and rat brain cDNAs, respectively, as probes. GABA-T seems to be a fairly ubiquitous enzyme in mammalian tissues and its function in these tissues with the exception of the brain could be largely metabolizing GABA into succinic semialdehyde for subsequent conversion into succinate, a source of metabolic fuel.

With the isolation of the cDNA, attempts were also made to map the gene on the chromosome using the technique of Fluorescence *in situ* Hybridization (FISH). This technique is widely used to localize and map mammalian genes and to detect chromosomal abnormalities (Trask, 1991; Lichter *et al.*, 1991). It involves the labeling of DNA probes, which are then denatured and hybridized to denatured chromosomes on microscopic slides. Unlike radiolabeling, the probes for FISH are fluoresceinated by random-primer labeling with fluorescent labeled dNTPs, for example, fluoresceine-dUTP. The labeled DNA probe and the target chromosomes are denatured and the complementary sequences allowed to reanneal. After annnealing, the slides are washed and the chromosomes counterstained and mounted for viewing under the fluorescence microscope to locate the position of the fluorescent probe.

The GABA-T cDNA was random-primer labeled using the Prime-It Fluor Fluorescence Labeling Kit from Stratagene and the fluoresceinated probe denatured and used in hybridization reactions with denatured erythrocyte chromosomes in metaphase immobilized on microscope slides following the procedures outlined by the manufacturer in the protocol with the kit. Although some fluorescent spots were located on some of the chromosomes, there was a lot of background making it impossible to interpret the results. It is hoped that this work can be pursued in future experiments to locate the gene on the chromosome, in order to eventually detect any chromosomal abnormalities in people prone to epileptic seizures.

CHAPTER 3

MOLECULAR CLONING AND EXPRESSION OF HUMAN BRAIN cDNA ENCODING 4-AMINOBUTYRATE AMINOTRANSFERASE

INTRODUCTION

Several studies on 4-aminobutyrate aminotransferase (GABA-T), the enzyme that catalyzes the breakdown of the major inhibitory neurotransmitter, GABA, have been carried out using the enzyme purified from mammalian tissues. Some neurological diseases such as epilepsy have been associated with decreased concentrations of GABA, since compounds that increased the GABA concentration by inactivating GABA-T, have proven to be useful anti-convulsants (Tower, 1976; Fowler and John, 1972; Gibson *et al.*, 1990). Although tissues from mammals such as rat, mouse, rabbit and pig can be obtained relatively easily, facilitating the study of the enzyme from these sources, the attendant problems involved with the procurement of human tissues in amounts and/or the state for purification of the enzyme have limited studies on the human enzyme. In order to carry out extensive studies on the interaction of new drugs and help in the design of novel drugs to manage neurological diseases where GABA-T action has been implicated, it is imperative that the enzyme be made more available and in quantities large enough to support such studies.

The expression of heterologous genes in bacterial cells has been used for high-level production of numerous prokaryotic and eukaryotic proteins. As the cDNA for hGABA-T has been isolated, this portion of the thesis is designed to clone and express the hGABA-T gene in *E. coli* cells using the pET expression system (Novagen). This is

being carried out in order to obtain the human enzyme more readily and in larger quantities to facilitate more studies on the enzyme.

Two expression vectors, pET30a and pET12a, were employed in separate experiments for the subcloning of hGABA-T cDNA and subsequent expression in the bacterial host, BL21(DE3). Insoluble protein was obtained which was solubilized by urea and refolded, but enzyme activity was absent. Since chaperones have been shown to increase the yield of correctly folded, soluble active proteins both *in vitro* and *in vivo* (Mendoza *et al.*, 1992; Schröder *et al.*, 1993), co-expression of the target DNA and the co-chaperones, GroEL/ES, was attempted, but this also resulted in the production of an insoluble enzyme.

EXPERIMENTAL PROCEDURES

Materials

pET30a and pET12a expression vectors and *E. coli* expression host cells, BL21(DE3), from Novagen, Inc., Madison, Wisconsin, were gifts from Drs. Neil Quigley (Zoology Department) and Cynthia Peterson (Biochemistry Department), University of Tennessee, Knoxville. GroEL/ES plasmid was a gift from Dr. Frank Larimer, Biology Division, Oak Ridge National Laboratory. Perfect Protein Western Markers, S-Tag Western Blot Kit and His-Bind Resin and Buffer Kit were purchased from Novagen, Inc., Madison, Wisconsin. Restriction enzymes, calf intestinal alkaline phosphatase (CIP) and DNA ligase were purchased from New England Biolabs (NEB) Inc., Beverly, Massachussetts. Antibiotics, isopropyl-β-D-thiogalactopyranoside

(IPTG) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Company, St. Louis, Missouri.

Cloning of hGABA-T cDNA into the pET expression system

pET 30a vector (10 μg), was digested with B*am* HI (20units) in a total volume of 100 μl using NEB buffer #3 [50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT (pH 7.9 at 25°C)]. Digestion was carried out at 37°C for 2 h. To check for completion of the digestion, a 10 μl aliquot was taken, heated at 65°C for 20 min to stop the reaction, and 2 μl of 6X loading buffer were added. The sample was electrophoresed on 0.8% agarose at 25 mAmps for 2.5 h, stained in 0.01% ethidium bromide solution and viewed under UV light to locate the bands. Another 10μl aliquot of the completely digested sample was saved for later use in the ligation reaction as a control. An equal volume, (80 μl), of a 1/10 dilution of NEB buffer #3 was added to the digested vector DNA to give a final concentration of 0.5 μg/10μl, and 1 μl of calf intestinal alkaline phosphatase (CIP) (10units) was added and incubated at 37°C for 1 h. The reaction was stopped by addition of EDTA to a final concentration of 5 mM, and the mixture was heated at 75°C for 10 min to inactivate CIP. The reaction mixture was cooled to room temperature, and the dephosphorylated DNA was isolated by ethanol precipitation. The DNA was dissolved in 10 mM TE buffer pH 8.0 and used in ligation reactions.

The B*am* HI-digested fragment of hGABA-T cDNA comprising the ORF was ligated into the B*am* HI digested and dephosphorylated vector DNA . The following reactions were set up:

i. B*am* HI digested/dephosphorylated vector DNA plus B*am* HI fragment of hGABA-T cDNA in the ratio 1:1 and 1:2

ii. Digested/dephosphorylated vector DNA only

iii. Digested vector not dephosphorylated

iv. Fragment only

in a total reaction volume of 10 µl containing 1X ligase buffer [50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10mM DTT, 1 mM ATP, 25 µg/ml bovine serum albumin] and 0.5 µl of ligase (200 units) per tube and incubated at 16°C overnight. The ligase reaction was stopped by heating at 65°C for 20 min, and aliquots of the mixture were used in transformation reactions.

Competent XLI-Blue cells were prepared following the standard protocol described in Sambrook *et. al.*, (1989). Transformation reactions were set up by pipetting 200 µl of competent XLI-Blue cells into chilled Falcon tubes using chilled pipette tips. Ligation mixtures and uncut vector equivalent to 40-50 ng DNA were added separately to each tube, swirled gently to mix and incubated on ice for 30 min. The tubes were then transferred into a water-bath at 42°C, heat-shocked for 90 secs and quickly returned to the ice for another 2 min. 800 µl of SOC culture media were added to each tube and incubated at 37°C with gentle shaking for the cells to express antibiotic resistance. Aliquots of the cell culture were taken after the incubation and plated on LB plates containing kanamycin (30 µg/ml) and $MgSO_4$ (20 mM). As a control, competent cells were plated on LB agar plates with or without kanamycin. The plates were incubated overnight at 37°C. Colonies were picked from the plates and inoculated into LB/kanamycin media and incubated at 37°C with constant shaking. Duplicate plates were prepared by inoculating fresh LB/agar/kanamycin plates with the same colonies used in inoculating the liquid media. Transformants were picked, and recombinant plasmid DNAs were purified using the Wizard miniprep kit, (Promega).

Plasmid DNA was digested with B*am* HI, S*al* I or E*co* RI in separate reactions to check for correctly oriented inserts and the possibility of tandem insertion.

pET12a was also digested with B*am* HI and used in ligation reactions with hGABA-T cDNA for subsequent transformation into XL1-Blue cells as described earlier. The antibiotic used in this case for plasmid maintenance in the cells was ampicillin at a concentration of 50 μg/ml. Insertion of the fragment in the correct orientation and the possibility of double insertions were determined by S*al* I and E*co* RI digestion of the recombinant gene, respectively. The recombinants with the insert in the correct orientation were purified and used to transform the expression host, BL21(DE3) competent cells. Recombinant DNAs were again purified from the transformed cells and digested once more with B*am* HI to confirm the insertion. Glycerol stocks were made for the positive clones for storage at -70°C.

Expression of hGABA-T in the host bacterial cells

Overnight cultures of transformed cells (pET30a.hGABA-T) in LB/kanamycin or (pET12a.hGABA-T) in LB/ampicillin media were used to inoculate fresh media in the ratio 1 : 100. The cultures were grown at 37°C with constant shaking until OD_{600} = 0.4-0.5. A portion of the culture was induced with IPTG, to a final concentration of 1 mM, for the pET30a recombinant, and 0.4 mM, for the pET12a recombinant. Incubation was continued at 37°C for up to 4 h, and aliquots taken at different time intervals were prepared for SDS-PAGE and Western blotting analyses to check for expression. Electrophoresis was carried out in 12% polyacrylamide gels under denaturing conditions at constant voltage (75-100 volts) for 2-3 h with Tris-glycine buffer (25 mM Tris, 250 mM glycine, 0.1% SDS) as the running buffer. For SDS-PAGE on whole cell extracts, cells from 100 μl cultures collected at different time

intervals by centrifugation were suspended in 40 µl 1 X SDS-PAGE sample buffer, boiled for 4 min in a boiling water-bath and applied to the gel in 10-15 µl volumes per well. Aliquots of cells were also resuspended in 50mM Tris HCl, pH 8.0, containing 2 mM EDTA. Corresponding small scale preparations of crude extracts were obtained by harvesting 1.5 ml aliquots of the cell culture at various time intervals. The cells were pelleted by centrifugation and resuspended in 25 µl of 0.1 M Tris-HCl buffer, pH 8.0. Lysozyme prepared in the same buffer was added to a concentration of 50 mg/ml, and the mixture was incubated at 37°C for 10 min. The cells were lysed by alternating 5 min periods of freezing in dry ice/ethanol and incubating at 37°C, repeated three times. The sample was centrifuged, and aliquots of the supernatant fraction were added to 2X SDS loading buffer and applied to a SDS-polyacrylamide gels for resolution and subsequent staining with Coomassie blue or for Western blotting to locate the expressed protein bands.

Control cultures with transformants carrying pET30a vector only were grown and treated in the same way for comparison with transformants carrying the recombinant vector. For larger culture volumes, the cells were pelleted by centrifugation at 5,000 x g for 5 min, and cell pellets were resuspended in 1/10 cell culture volume of 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM EDTA and 1 mM β-mercaptoethanol (lysis buffer). Lysozyme was added to a final concentration of 100 µg/ml followed by addition of 1% Triton X-100 (1/10 culture volume) and PMSF to a final concentration of 1 mM. The sample was incubated at 30°C for 15 min. At the end of the incubation period, the tube was placed in an ice bath to chill, and the cells were lysed by sonicating with a Branson Sonifier at the high output setting for three to five 10 sec pulses, with a 2 min interval between each cycle. Cell lysates were fractionated by centrifugation at 12,000 x g for 5 min at 4 °C. Aliquots of the

supernatant were checked for the presence of the protein by SDS-PAGE, followed by Coomasie blue staining and Western blot analysis. The pellet was rinsed 2X by resuspending it in fresh lysis buffer and centrifuged as above. Different compositions of the lysis buffer were also prepared by addition of NaCl, sucrose or PLP to see if such treatments would help in solubilization of the protein.

Effect of temperature and salt concentration on expression

The cell cultures were grown to an OD_{600} = 0.4-0.5 and incubated with constant shaking at 18°C, 25°C, 30°C or 37°C following IPTG induction to study the effect of temperature on expression. In addition, cells were grown at higher sodium chloride concentration (0.4 M) instead of the normal concentration of (0.17 M) in LB media at the various temperatures.

Western blotting

Aliquots of whole cell lysates, soluble and the insoluble fractions after lysozyme treatment, sonication and centrifugation were electrophoresed on a 12% polyacrylamide/SDS gel together with Perfect Protein Western Markers. Following electrophoresis, the samples were transferred electrophoretically onto Immobilon-P transfer membrane (Millipore, Bedford, Massachusetts, USA) using the Genie Electroblotter apparatus from Idea Scientific Company, Minneapolis, MN. After the transfer, the membrane was removed from the blotting apparatus and incubated in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) containing 1% Gelatin at room temperature for 15 min to block excess protein binding sites. The membrane was then incubated in 1/5,000 dilution of S-Protein Alkaline Phosphatase Conjugate in TBST for another 15 min and rinsed 4 times in TBST within a period of 1-2 min by adding the solution, briefly shaking and decanting. A solution made up of

60 µl each of Nitro Blue Tetrazolium (NBT) and Bromo-chloro-indolyl phosphate (BCIP) in 15 ml Alkaline phosphatase (AP) buffer [100 mM NaCl and 5 mM $MgCl_2$ in 100 mM Tris-HCl, pH 9.5] was added, and the membrane was incubated with the substrate solution at room temperature for the color to develop. The blot was rinsed in deionized water, air-dried and stored wrapped in aluminum foil at room temperature.

Purification of Expressed Protein on His.Bind Column

The procedure outlined in the pET System Manual for rapid affinity purification of the expressed protein on the His-Bind Ni^{2+} resin was followed. The column (2.5 ml settled resin volume/100 ml culture) was allowed to pack under gravity and rinsed with 3 volumes of sterile deionized water, followed by 5 volumes of 1X Charge Buffer (50 mM $NiSO_4$ in 20 mM Tris-HCl buffer, pH 7.9) and finally with 3 volumes of 1X Binding Buffer (5 mM imidazole, 0.5 M NaCl in 20 mM Tris-HCl, pH 7.9) containing 6 M urea. The cell extract was prepared by resuspending the harvested cells in ice-cold 1X Binding Buffer (40 ml/100 ml cell culture volume) and sonicating in an ice-bath to break up the cells and shear the DNA. The lysate was centrifuged at 20,000 x g for 15 min at 4°C to pellet the insoluble protein and cellular debris. The supernatant was decanted, and the precipitate was resuspended in 20 ml 1X Binding Buffer and briefly sonicated and centrifuged as above. The pellet was resuspended in 5 ml 1X Binding Buffer containing 6 M urea, pH 7.9 and incubated on ice for 1 h to dissolve the protein completely. The sample was centrifuged at 39,000 x g for 20 min, and the supernatant filtered through a 0.45 micron membrane and loaded on the column.

The column was washed with 10X the column volume of 1X Binding Buffer containing 6 M urea, pH 7.9, followed by a second wash with 15 ml of 20 mM imidazole buffer [a mixture of 11 ml 1X Binding Buffer and 4.1 ml 1X Wash Buffer

(60 mM imidazole, 0.5 M NaCl, in 20 mM Tris- HCl, pH 7.9)] containing 6 M urea. The protein was then eluted with 1X Elute Buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) containing 6 M urea. One ml fractions of the eluted protein were collected and the purification was monitored by running the aliquots of the fractions on SDS-polyacrylamide gels for Coomassie blue staining and Western blotting.

Thrombin digestion of soluble protein

The protein was initially solubilized in 20mM Tris-HCl buffer, pH 8.4, containing 6 M urea, 150 mM NaCl and 2.5 mM $CaCl_2$, and incubated for 1 h on ice for complete solubilization. The sample was diluted 1/3 by addition of 2X the sample volume of the above buffer without urea, thus bringing the urea concentration to 2 M. The sample was centrifuged to pellet any insoluble material, and an aliquot of the supernatant fraction was separated by SDS-PAGE to confirm the presence of the protein. Different concentrations of thrombin were added to separate sample tubes and incubated at room temperature for a total of 4 h. The success of digestion was monitored by SDS-PAGE. Digested samples were applied to the His-Bind affinity column for purification, following the protocol described previously, with all the solutions containing 2 M urea.

Co-expression of pET12a.hGABA-T and GroEL/ES in BL21(DE3)

The expression vector BL21(DE3) containing either the recombinant gene, pET12a.hGABA-T or the plasmid for GroEL/ES were made competent following standard methods. The competent cells containing GroEL/ES were transformed with the recombinant gene, pET12a.hGABA-T, while those containing the recombinant gene were transformed with GroEL/ES plasmid DNA. The transformed cells were plated on LB agar plates containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol and

incubated overnight at 37°C. Several colonies were picked and grown in LB containing both antibiotics at the concentrations stated. Plasmid DNAs were isolated and digested with E*co* RI to determine the success of the transformation.

RESULTS AND DISCUSSION

Cloning of hGABA-T into the pET expression system

The fragment obtained after B*am* HI digestion of pBluescript, consisting of the open reading frame of hGABA-T cDNA used in the Northern blot experiment was also used in the ligation reactions. The enzyme cuts at two positions within the cDNA sequence, at 168 and 1633 bp, yielding a fragment containing all of the mature sequence for hGABA-T, with the loss of most of the mitochondrial presequence (Appendix A2.1). Evaluation of the pET vector maps (Appendices A1.1&A1.2) and the fragment ends generated by B*am* HI digestion of the fragment indicated that the vector containing the cloning site in reading frame designated as **"a"** should be used for insertion of the GABA-T cDNA in the correct reading frame for expression. The initial subcloning was done in a recA$^-$ cloning strain, XL1-Blue.

Out of seven different colonies picked after the ligation and transformation reactions, six had the insert, as determined by digestion of the purified plasmid DNAs with B*am* HI, Fig. 3.1. Lanes 1-4 and 6-7 were loaded with DNA isolated from colonies transformed with the vector/hGABA-T cDNA ligation mixture. Results of the digestion revealed that there was no insert in the sample run in lane 4 as the size of the DNA was the same as that of the digested pET30a with no insert (lane 5). This indicates that the

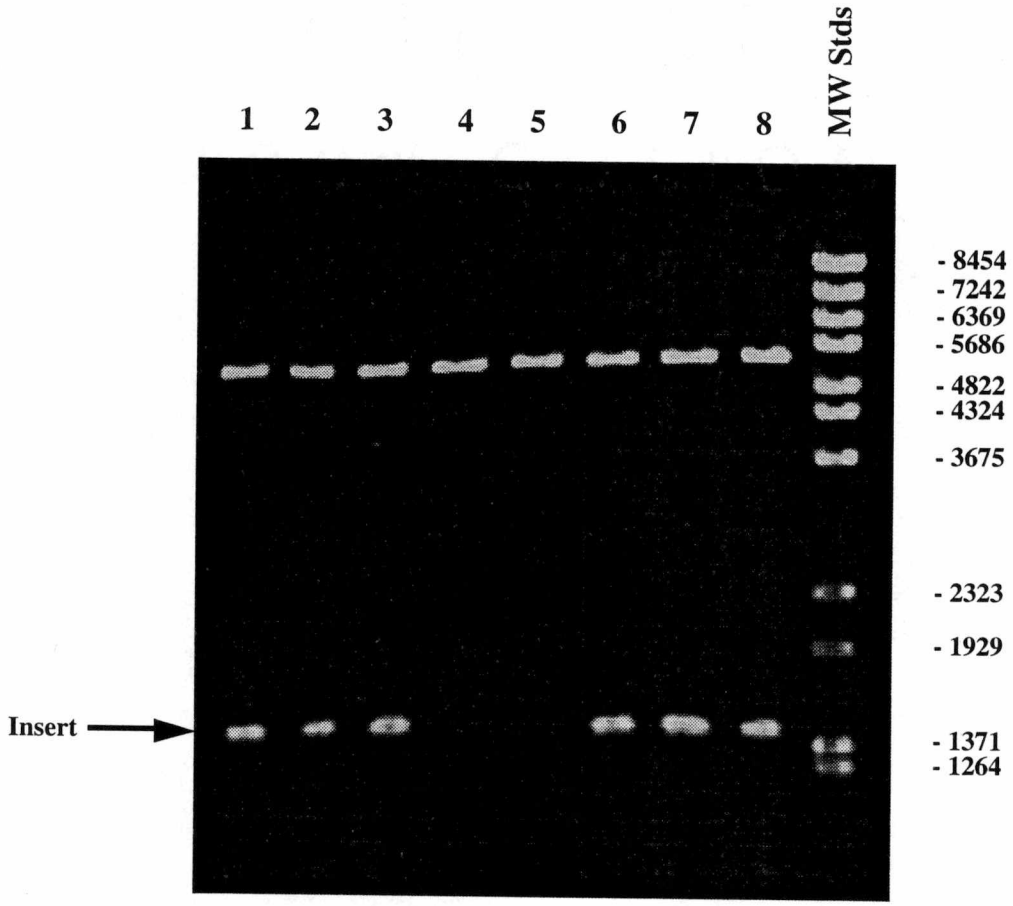


Fig. 3.1 : B*am* HI digestion of pET 30a +/- hGABA-T established in XL1-Blue.

Lanes 1-4, 6-8 - digested DNA from cells transformed with pET 30a-hGABA-T cDNA

Lane 5 - digested DNA from cells transformed with pET 30a only

colony had unligated vector DNA, implying that self-ligation of the cut plasmid had occurred and that the dephosphorylation was not 100% complete. From the number of transformants picked, the ligation reaction appeared to have a success rate of about 85%.

To test for the correct orientation for the insertion, the recombinant DNA was digested with S*al* I, as inspection of the recombinant restriction map (Appendix A3.1) indicated that, when digested with *Sal* I, DNA inserted in the correct orientation should give a fragment about the same size as the inserted fragment and another one the size of the vector. cDNA inserts in the wrong orientation (Appendix A3.2) should give one fragment about 7.0 kbp and a smaller fragment of 52 bp, which is undetectable on the gel. The results for S*al* I digestion shown in Fig. 3.2 indicated that four (lanes 3,4,7&9) out of the six colonies picked had the insert in the correct orientation, while the other two (lanes2&8) were incorrectly oriented. Since the insertion of the fragment was directed at only one restriction site, it is not surprising that insertion in the wrong orientation was observed, especially when it was detected from the B*am* HI digestion that the dephosphorylation was not 100% successful. These results also confirmed the absence of the insert found earlier on for one of the colonies for B*am* HI digestion, since the same sample in lane 5 gave one band, the size of the vector, with S*al* I digestion.

Fig. 3.3 shows the results obtained for the digestion of the DNAs with E*co* RI to test for the presence of tandems. The results showed that there were no double insertions, as E*co* RI cuts at only one point on the vector and the size of the recombinant DNAs was around 7.0 kbp, agreeing with the calculated value for the recombinant DNA and confirming the presence or absence of the insert.

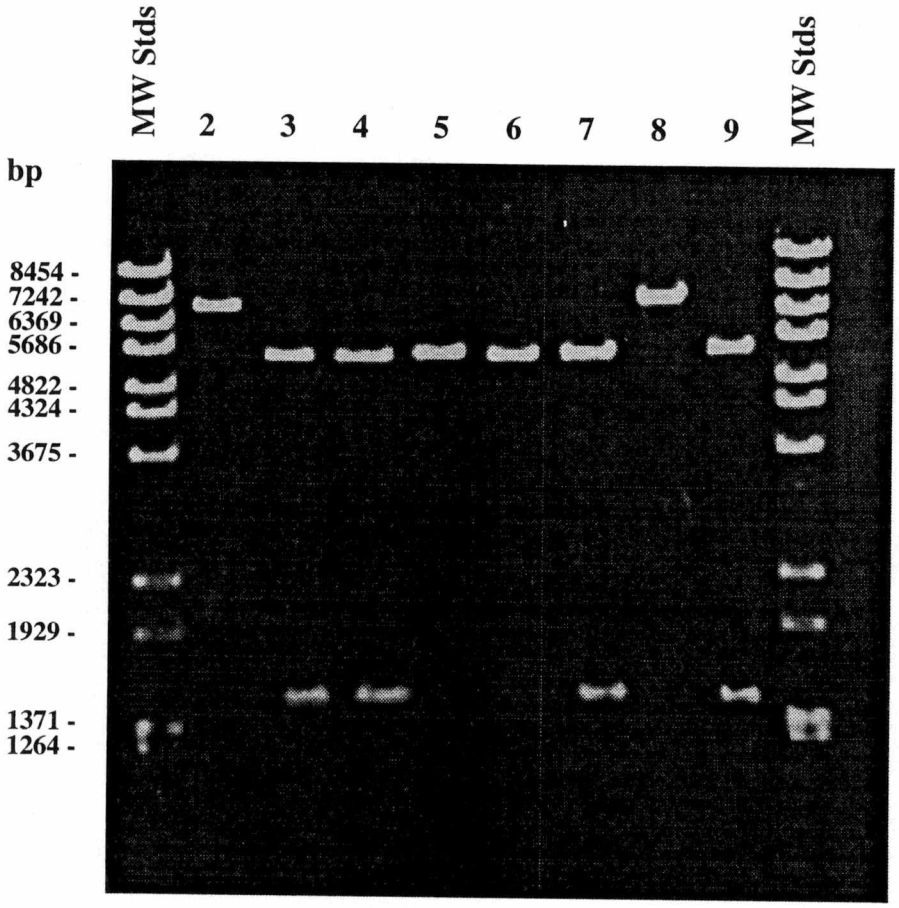


Fig. 3.2 : *Sal* I digestion of pET30a +/- hGABA-T cDNA established in XL1-Blue

Lanes 2-5, 7-9 - Digested DNA from cells transformed with pET30a-hGABA-T

Lane 6 - Digested DNA from cells transformed with pET30a only

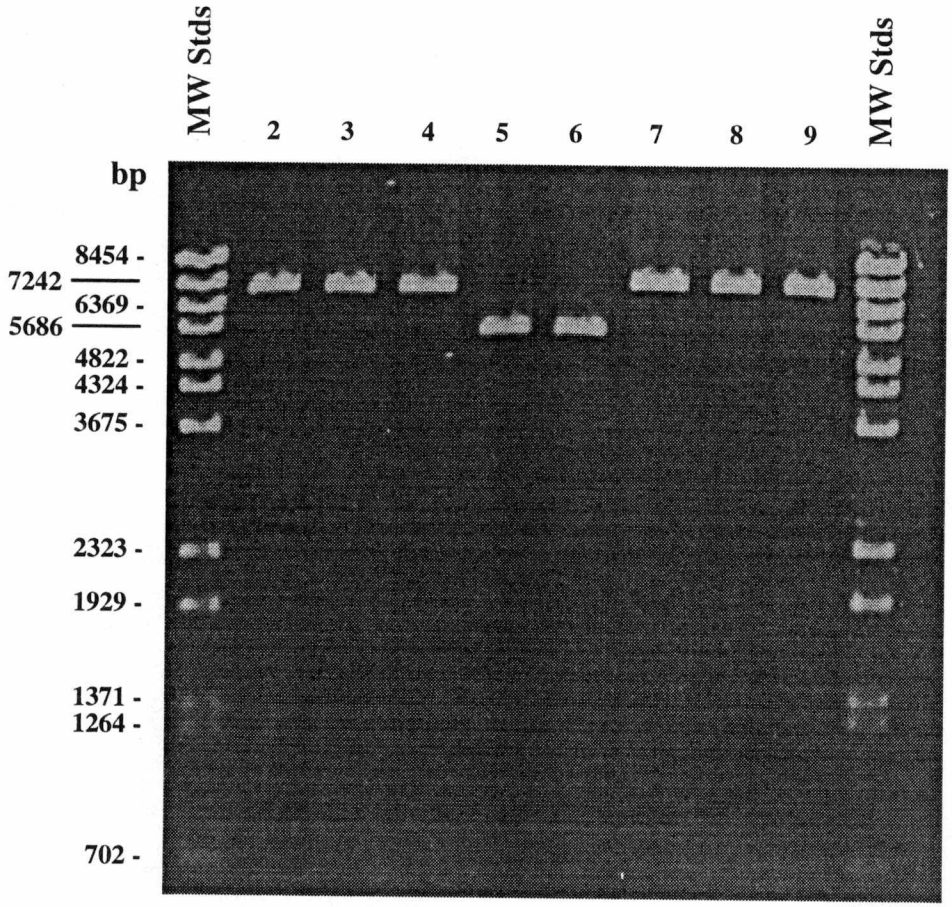


Fig. 3.3 : E*co* RI digestion of pET30a +/- hGABA-T cDNA established in XL1-Blue.

Lanes 2-5, 7-9 - digested DNA from cells transformed with pET30a+hGABA-T
Lane 6 - digested DNA from cells transformed with pET30a only

Out of seven colonies transformed with the pET12a/hGABA-T recombinant DNA, only three had the insert indicated after B*am* HI digestion (Fig. 3.4). S*al* I digestion indicated that, two out of these three had the insertion in the correct orientation, as shown in Fig. 3.5. It was deduced from the vector map shown in Appendix A3.3 that S*al* I digestion on the plasmid with the insert in the correct orientation should give a fragment the size of the vector plus insert (about 6.07 kbp) and a smaller one of 40 bp, which could not be detected on the gel. Those plasmids containing inserts in the wrong orientation should give two fragments, one about the size of the insert and the other about that of the vector, (Appendix A3.4). Results of E*co* RI digestion are shown in Fig. 3.6. All of the samples showed single bands, with sizes of the DNA in the first three lanes corresponding to that of the plasmid plus insert, while the others gave bands corresponding to the vector size. Since E*co RI* cuts at one site on the vector and does not cut the insert DNA, these results confirmed the absence of double insertion and also the presence of the insert in the three colonies picked from B*am* HI digestion.

The purified DNAs for the correct recombinants and those from the vectors were used to transform the expression host, BL21(DE3), and once again the transformants were picked and grown in LB with the appropriate antibiotic and the DNA purified and digested with B*am* HI to confirm the insertion. Fig. 3.7 shows the results obtained, confirming the presence of the recombinant plasmid in the expression host. One of the recombinants was selected and the DNA partially sequenced using the T7 promoter primer. The results obtained confirmed the insertion in the correct orientation, implying that the cloning had been successful.

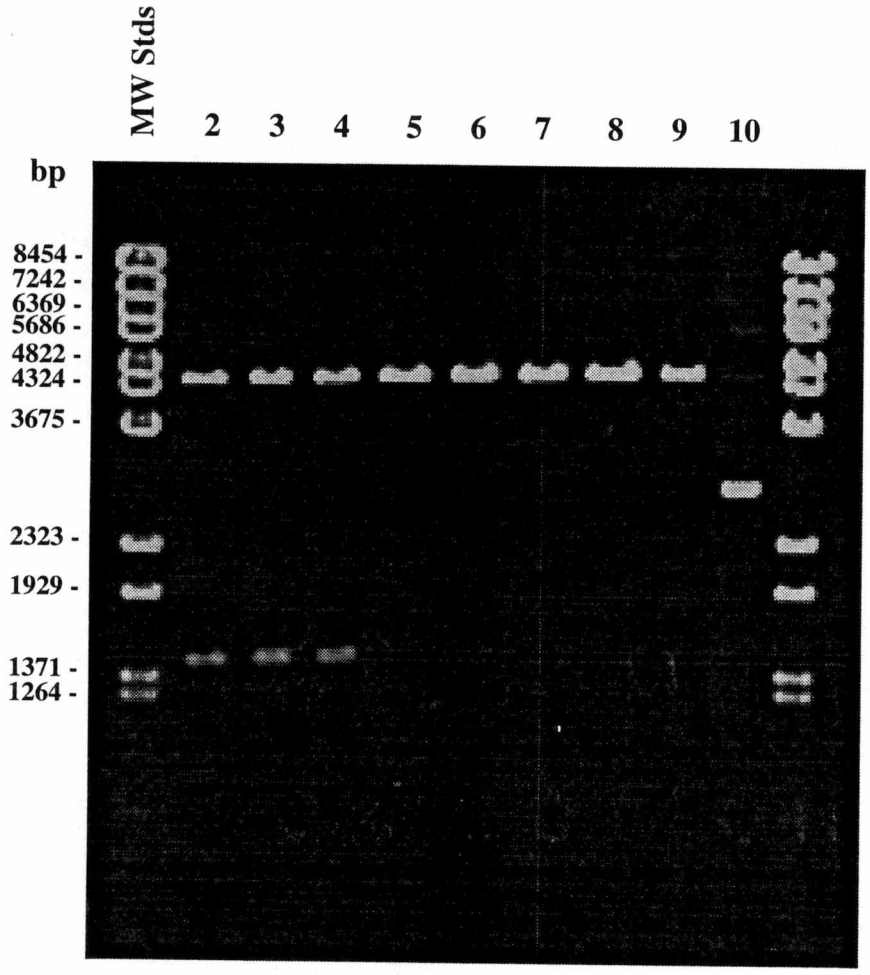


Fig. 3.4 : B*am* HI digestion of pET 12a +/- hGABA-T established in XL1-Blue.

Lanes 2-8 - digested DNA from cells transformed with pET12a-hGABA-T
Lane 9 - digested DNA from cells transformed with pET12a only
Lane 10 - undigested pET12a

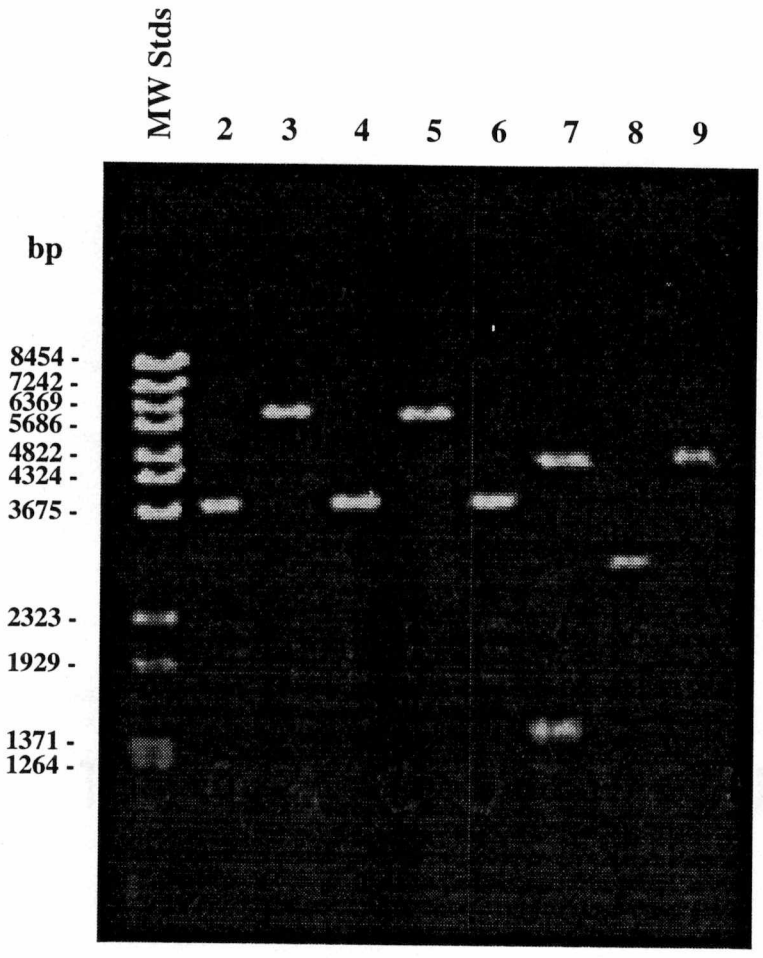


Fig. 3.5 : *Sal* I digestion of pET 12a +/- hGABA-T cDNA

Lanes 2, 4, 6 - Undigested DNA from cells transformed with pET 12a hGABA-T cDNA

Lanes 3, 5, 7 - Digested DNA from cells transformed with pET 12a hGABA-T cDNA

Lane 8 - undigested DNA from cells transformed with pET 12a only

Lane 9 - digested DNA from cells transformed with pET 12a only

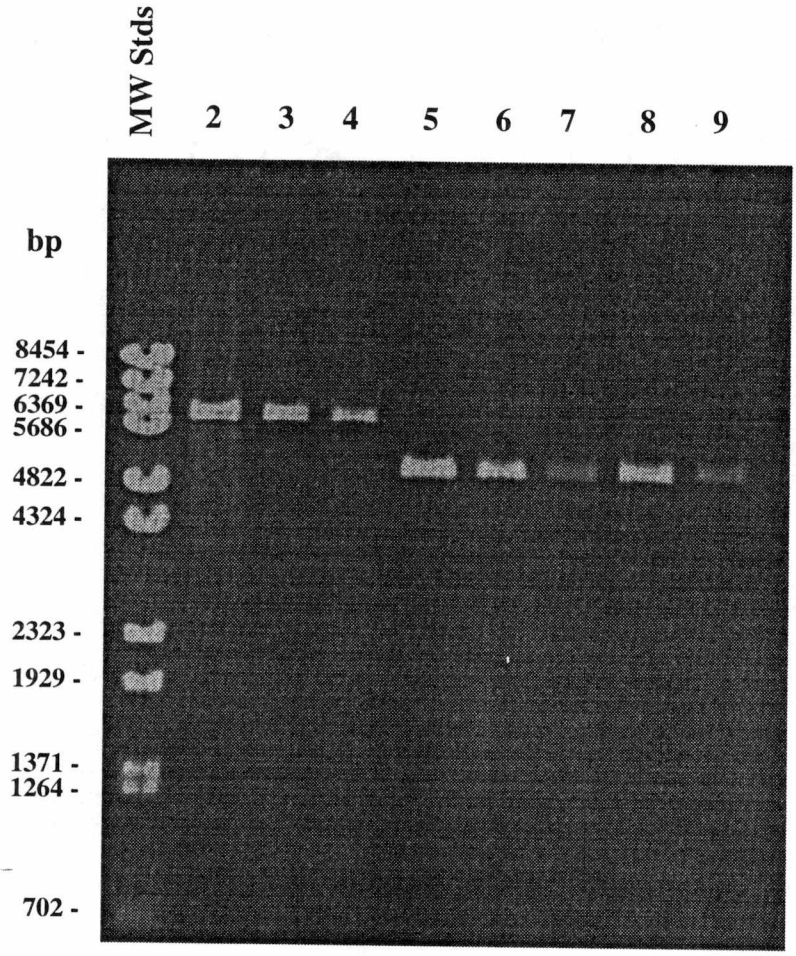


Fig. 3.6 : E*co* RI digestion of pET12a +/- hGABA-T cDNA established in XL1-Blue.

Lanes 2-8 - digested DNA from cells transformed with pET12a-hGABA-T cDNA

Lane 9 - digested DNA from cells transformed with pET12a only

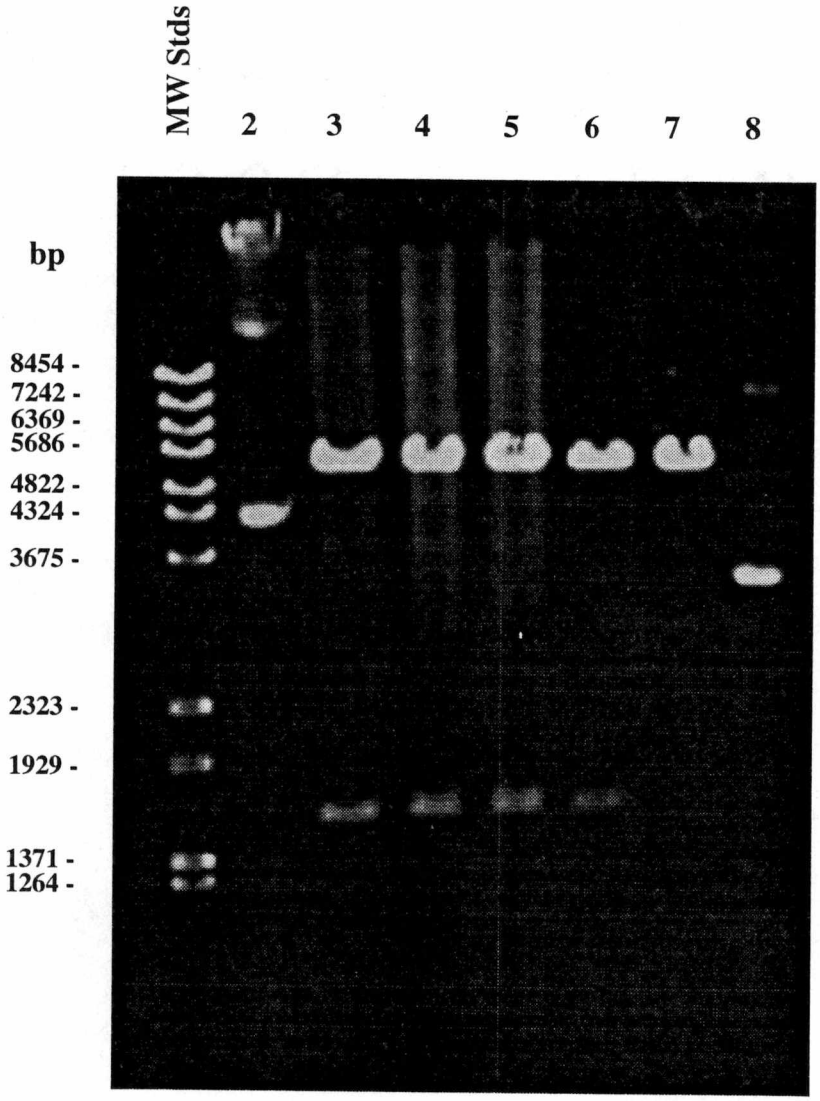


Fig. 3.7 : B*am* HI digestion of pET30a +/- h GABA-T cDNA established in BL21 (DE3).

Lane 2 - undigested DNA from cells transformed with pET30a + hGABA-T cDNA
Lanes 3-6 - digested DNA from cells transformed with pET30a + hGABA-T cDNA
Lane 7 - digested DNA from cells transformed with pET30a only
Lane 8 - undigested DNA from cells transformed with pET30a only

Results of *Eco* RI digestion of plasmid DNA purified from cells transformed with both GroEL/ES and pET12a.hGABA-T recombinant plasmid are shown in Fig. 3.8. The enzyme cuts at two sites on the GroEL/ES plasmid and only at one site on the recombinant DNA. From the results, two samples clearly gave three bands upon digestion: a larger band corresponding to pET12a.hGABA-T and two other bands corresponding to the GroEL/ES plasmid. One of the samples showed three bands, the two smaller bands corresponding to the GroEL/ES plasmid and a larger band corresponding to the size of the vector with no insert. It was observed that the transformation was more successful starting with competent cells containing GroEL/ES plasmid and transforming with the recombinant pET12a.hGABA-T than vice versa.

Expression and detection of hGABA-T subcloned in pET30a

Figs. 3.9&3.10 show the time course of expression after IPTG induction detected by SDS-PAGE followed by Coomassie blue staining and Western Blot analyses, respectively, on whole cell lysates. An intense protein band of about 56 kDa in molecular weight was observed after IPTG induction for the cells transformed with the recombinant gene, while the band was absent in the uninduced cells or in those containing only the vector, pET30a. The size of the band agreed with the expected molecular weight computed for the fusion protein, with the intensity of the band increasing with increase in time after the induction, indicating that the protein was being expressed and that the amount of protein expressed increased with increase in time after induction. Western Blot analysis confirmed this observation, but revealed several protein bands with lower molecular weights on the blot, indicating that there was degradation of the expressed protein. The degradation occured as early as 30 min of incubation (Fig. 3.10) and at the lower temperatures as well. Growth of the induced

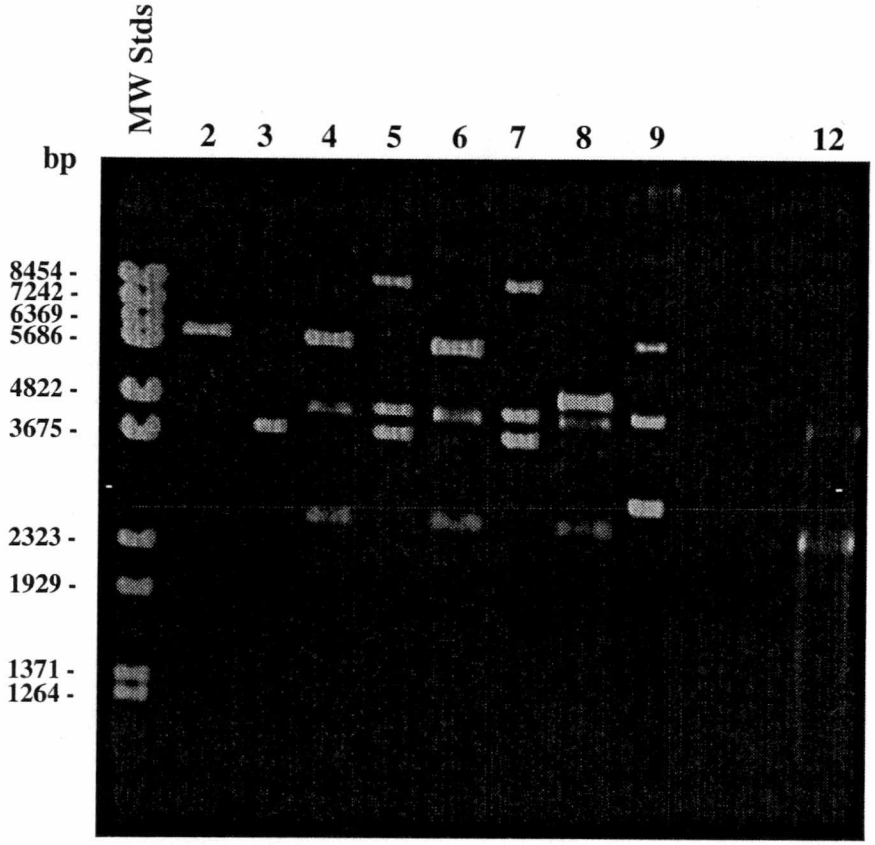


Fig. 3.8 : *Eco* RI digestion of pET12-hGABA-T +/- GroEL/ES plasmid

Lane(s) 2 - Digested pET12a-hGABA-T cDNA
3 - undigested pET12a-hGABA-T cDNA
4,6,8 - digested pET12a-hGABA-T cDNA + GroEL/ES
5,7,9 - undigested pET12a-hGABA-T cDNA + GroEL/ES
12 - digested GroEL/ES

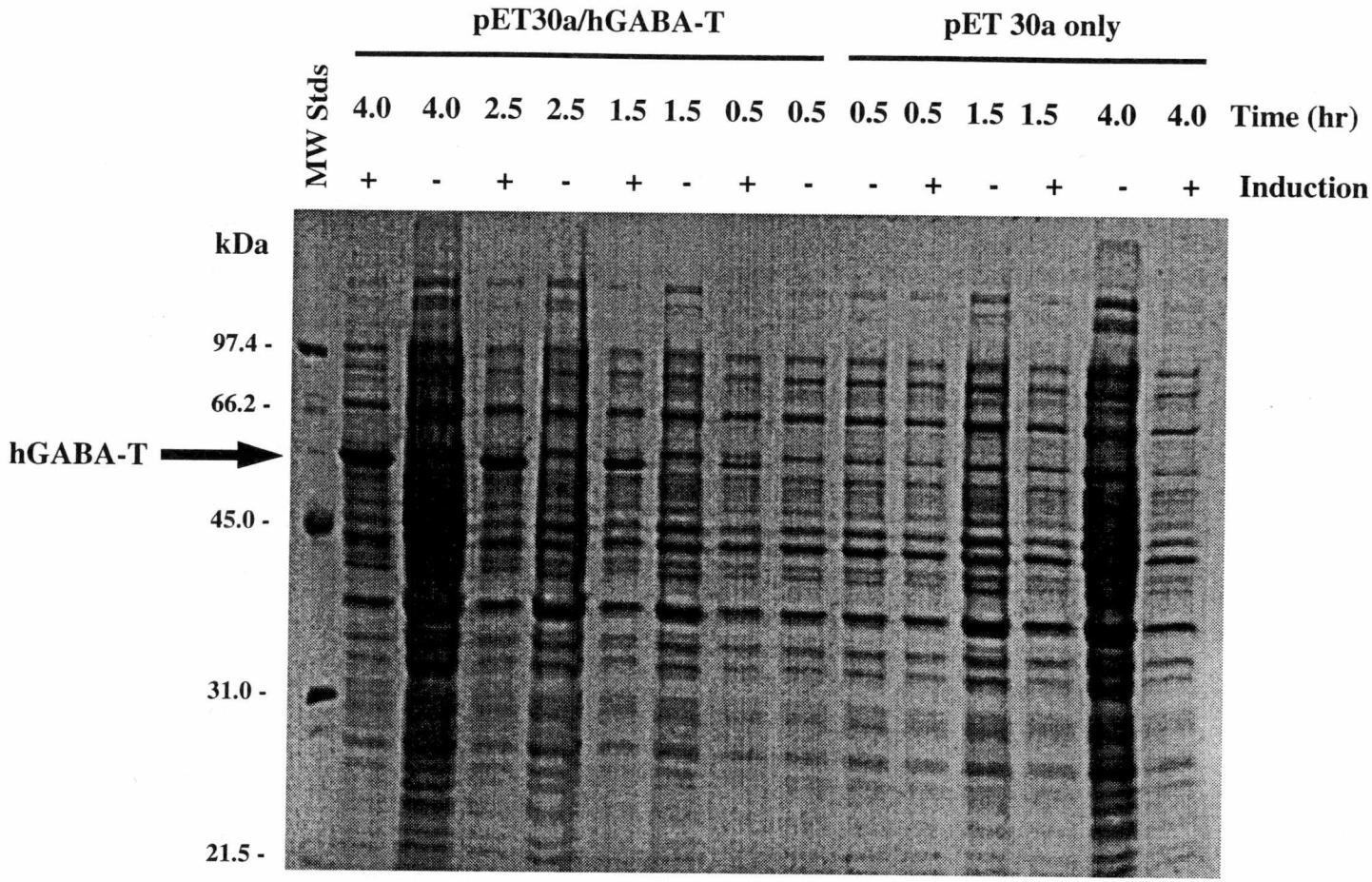


Fig. 3.9 : Induction time course of hGABA-T fusion protein in BL21(DE3) cells transformed with pET30a-hGABA-T.

Aliquots of cell culture were removed at the indicated times after IPTG addition to induce expression. Samples were treated as described in the "experimental procedures" for SDS-PAGE and stained with Coomassie blue. The target fusion protein is indicated by the arrow.

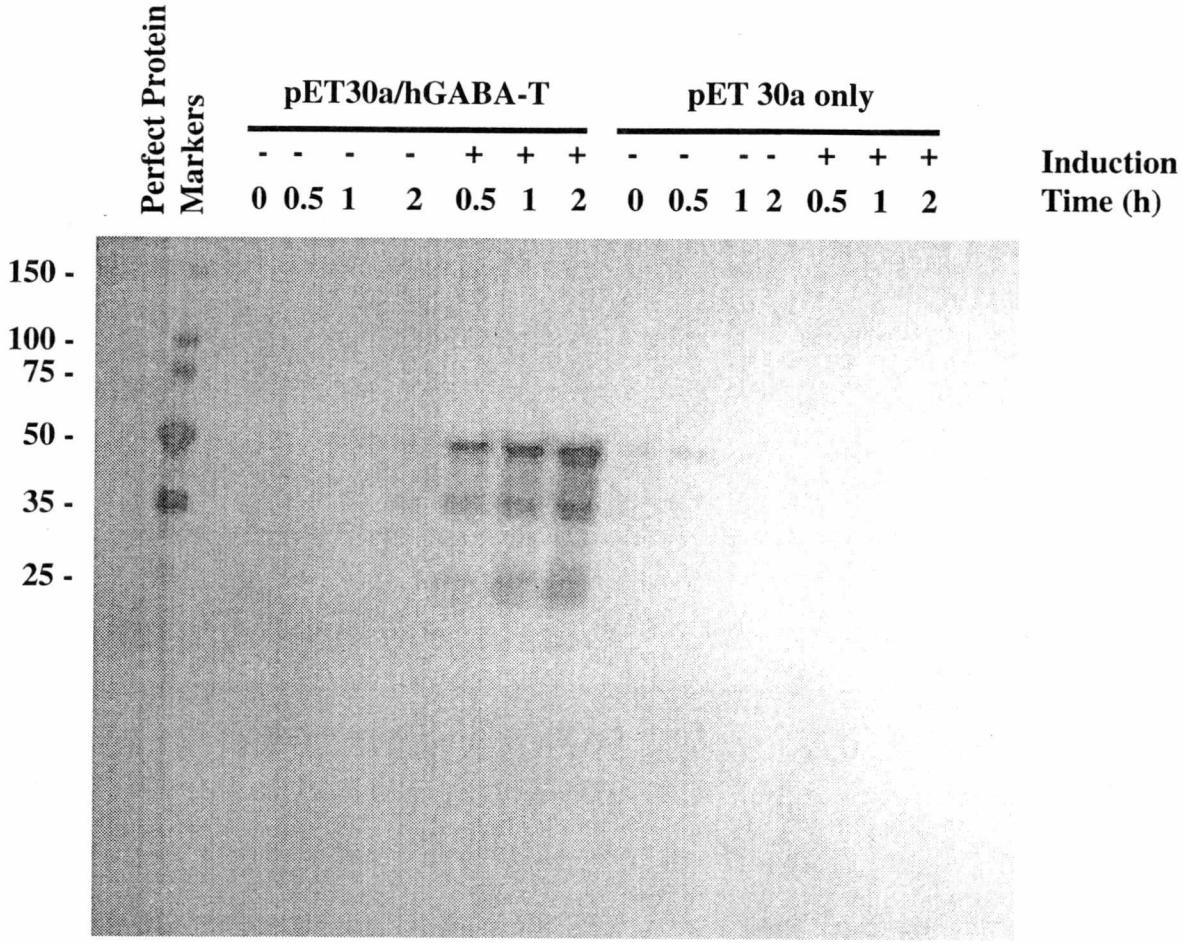


Fig. 3.10 : Induction time course of hGABA-T expressed as S-Tag fusion protein in pET 30a followed by Western blotting on whole cell lysate.

Cells were lysed and treated as described in the "experimental procedures".

cells was much slower and remained constant after about 3 h compared to the uninduced cells which grew rather profusely, especially at higher temperatures.

SDS-PAGE of whole cell lysates and the supernatant fractions after lysis of the harvested cells by sonication, followed by centrifugation, showed the expressed protein present in the whole cell lysate but absent in the supernatant fraction. Resuspension of the resultant precipitate in 1X SDS-PAGE sample buffer for electrophoresis, followed by Western blot analysis, confirmed the presence of the expressed protein in the precipitate (Fig.3.11).

In order to check whether the observed insolubility of the recombinant protein was the result of unsuccessful sonication, aliquots of subsequent lysate prepared under sterile conditions were plated on LB/kanamycin and LB agar plates without antibiotics. After incubation overnight at 37°C, there was no growth observed on either plate, implying that there was complete lysis and confirming the interpretation that the expressed protein was insoluble.

Accumulation of expressed protein in an insoluble state has been observed in a majority of cases (Harris,1983). A number of eukaryotic polypeptides expressed in *E. coli* directly, e.g. bovine and salmon growth hormones (George *et al.*, 1985; Sekin *et al.*, 1985), and apolipoprotein E (Vogel *et al.*, 1985), or as fusion proteins, e.g. insulin A and B chains (Goeddel *et al.*, 1979), and myoglobin (Varadarajan *et al.*, 1985), have been shown to exist as aggregates or inclusion bodies. It appears that expression of hGABA-T as an insoluble protein is a "normal" phenomenon observed in the expression of many other proteins.

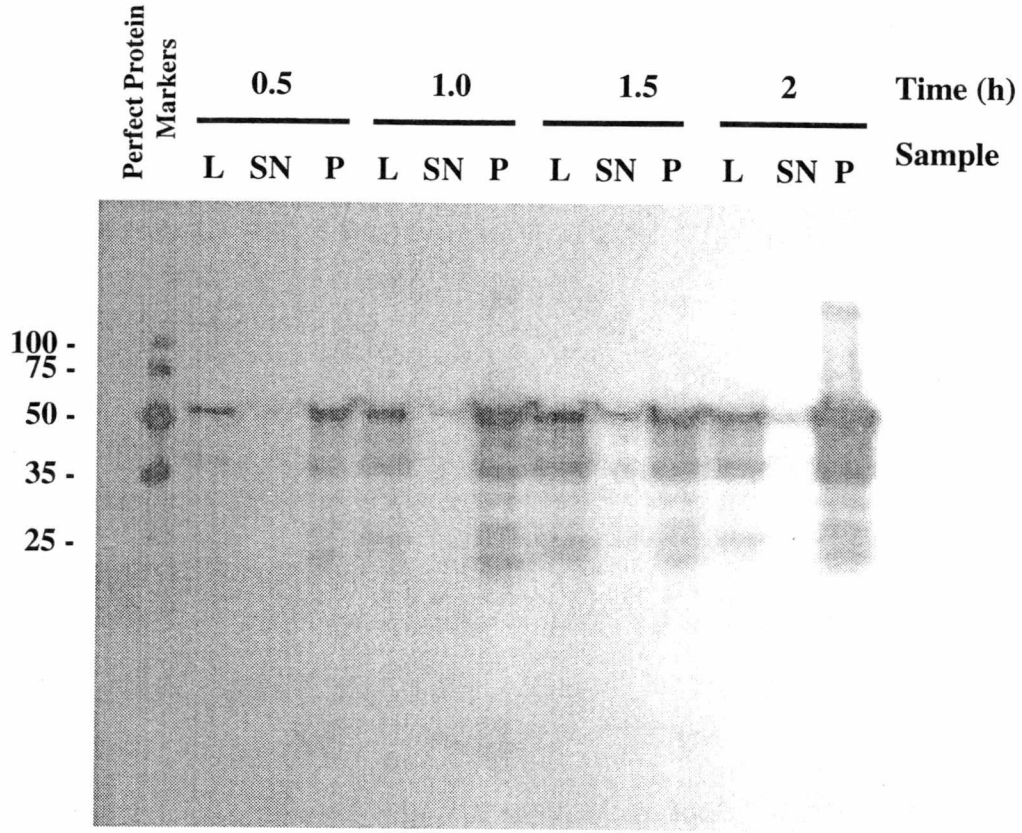


Fig. 3.11 : Induction time course of hGABA-T expressed as S-Tag fusion protein followed by Western blot on whole cell lysate (L), supernatant (SN), and precipitate (P).

Aliquots of cell culture were taken at the times indicated after IPTG induction. The samples were treated for SDS-PAGE and Western blot analysis as described in the "experimental procedures".

Even well before the advent of gene cloning, it had been observed that artificially induced abnormal proteins, also considered as "foreign" to the cells producing them, often accumulate in an insoluble state (Prouty, *et al.*, 1975). Subsequent to this, it was also found that even normal *E. coli.* proteins synthesized to high levels using recombinant techniques could also accumulate in insoluble forms (Gribskov and Burgess, 1983; Botterman and Zabeau, 1985) and within inclusion bodies (Cheng, 1983). Thus, insolubility of expressed proteins is not restricted to "foreign" proteins. A possible explanation for this phenomenon is that the expression of recombinant proteins may take place in an environment that is totally different in chemical properties such as redox potential and pH, in the type of proteases present, and in the types, specificities, and levels of the enzymes required for post-translational modification (Thatcher and Hitchcock, 1994). In addition, many foreign genes are engineered to produce proteins at levels that are much higher than the normal physiological levels and at a rate well above the cells capability for proper processing, thus resulting in their accumulation and the potential to induce unfavorable responses, including degradation of the protein by the host cells. Once degraded, the proteins tend to aggregate; therefore, the formation of the insoluble protein for the expressed protein could be a result of aggregation of the degraded protein.

Effect of temperature and salt concentration on expression of the protein

When cell cultures were incubated at different temperatures: 18°C, 25°C, 30°C or 37°C after IPTG induction and aliquots of the supernatant fractions after cell lysis were run on SDS-polyacrylamide gels for Coomassie blue staining and Western blot to visualize the protein bands, no striking difference was observed between the protein bands for the different samples. Expressed protein was absent in the supernatant

fractions, but present in the precipitates, implying that a change to lower temperature was ineffective in producing a soluble protein.

High growth temperatures as well as over-production of some proteins in *E. coli* cells have been shown to cause aggregation and misfolding, (Goldenberg et al., 1982; Haase-Pettingell and King, 1988; Goldberg and St John, 1976). A growth temperature of 25°C and short incubation times of 2-3 h were applied throughout the study for the expression of the protein, as lower temperature and shorter incubation periods have proven to give better results in many expression systems. Growth of cells at higher salt concentrations did not result in the expression of a soluble protein, however. High salt is also thought to induce the synthesis of heat shock proteins which could aid in the folding process. A combination of this and lower growth temperatures were used, but once again no soluble protein was obtained.

Solubilization of the insoluble protein

Since the above techniques failed to produce any soluble proteins, a method involving the use of the chaotropic reagent, urea, was applied to solubilize the expressed protein and refold it to determine whether a soluble and active protein could be obtained. Such procedures have been applied successfully to obtain active proteins originally expressed as insoluble aggregates, such as interferon-γ (Simons *et al.*, 1984) and salmon growth hormone (Sekine *et al.*, 1985). To dissolve the insoluble protein, pellets were rinsed twice with the lysis buffer containing either 0.1% Triton-X-100 or 0.5% Triton X-100 and 8% sucrose and solubilized in lysis buffer containing 6 M urea, followed by incubation on ice for at least 1 h to ensure complete dissolution. Initially, single-step dialysis was carried out by dialyzing in 50 mM potassium phosphate buffer, pH 7.4, containing BME (1 mM) and EDTA (2 mM), since this buffer is used in the

final purification of GABA-T from pig brain (Churchich and Moses, 1981). Since the recombinant protein was observed to precipitate in this buffer, stepwise dialysis was attempted, but precipitation of the protein was observed at a 2 M urea concentration. The dialysis buffer was changed to the same Tris-EDTA buffer, pH 8.0, containing BME used for lysis, with stepwise decreases in urea concentration, but precipitation was still observed at a 1 M urea concentration. Figs. 3.12A&B show the results obtained when the steps of the purification were followed by SDS-PAGE and Coomassie blue staining. It can be seen that increase in the concentration of Triton X-100 or addition of sucrose did not improve the solubility of the protein. Since GABA-T is a PLP-binding enzyme, it was speculated that the addition of PLP would help to refold the enzyme. In the subsequent solubilization and dialysis, PLP was added to a concentration of 0.1 mM and precipitation was once again observed at a 1 M urea concentration.

In some solubilization and refolding experiments reported on other proteins, higher concentrations of DTT or dithioerythritol (DTE) were used to obtain successful refolding (Buchner *et al.*, 1992). From the deduced amino acid sequence, mature hGABA-T was shown to possess 12 cysteine residues per monomer. Since the active enzyme has no disulfide bonds, the concentration of the reducing agent in the urea solubilized sample was increased to 50 mM DTT, and the sample was incubated for 1 h and dialyzed stepwise in Tris buffer, with decreasing urea concentrations until the buffer eventually contained no urea. The protein was completely soluble after this treatment, implying that a high concentration of the reducing agent, DTT, was required together with PLP for refolding. The results seem to suggest that aggregation could be the result of non-specific inter- and/or intra-chain disulfide bonds and that a high concentration of reducing agent was required to disrupt these interactions and to

Fig 3.12 : Purification of expressed hGABA-T

The steps of purification of the expressed protein were followed using two different washing procedures. Cells were harvested by centrifugation from equal volumes of cell culture after 2.5 h incubation after IPTG induction. One of the samples (A) was sonicated in 50 mM TE buffer (pH8.0) containing 1 mM β-mercaptoethanol, 0.1% Triton X-100. The other sample(B) was lysed in the same buffer containing 1 mM β-mercaptoethanol, 0.5% Triton X-100 and 8% sucrose. The supernatant fraction (1) was decanted and the insoluble fraction resuspended in the respective buffers described above and centrifuged. The supernatant fraction (2) was decanted after centrifugation to pellet the insoluble fraction. The rinsing process was repeated a third time resulting in the supernatant fraction (3). The resultant pellet (3) was then solubilized in 50 mM TE buffer (pH8.0) containing 6 M urea and 1 mM β-mercaptoethanol. Stepwise dialysis in decreasing concentrations of urea in 50 mM TE buffer was carried out.

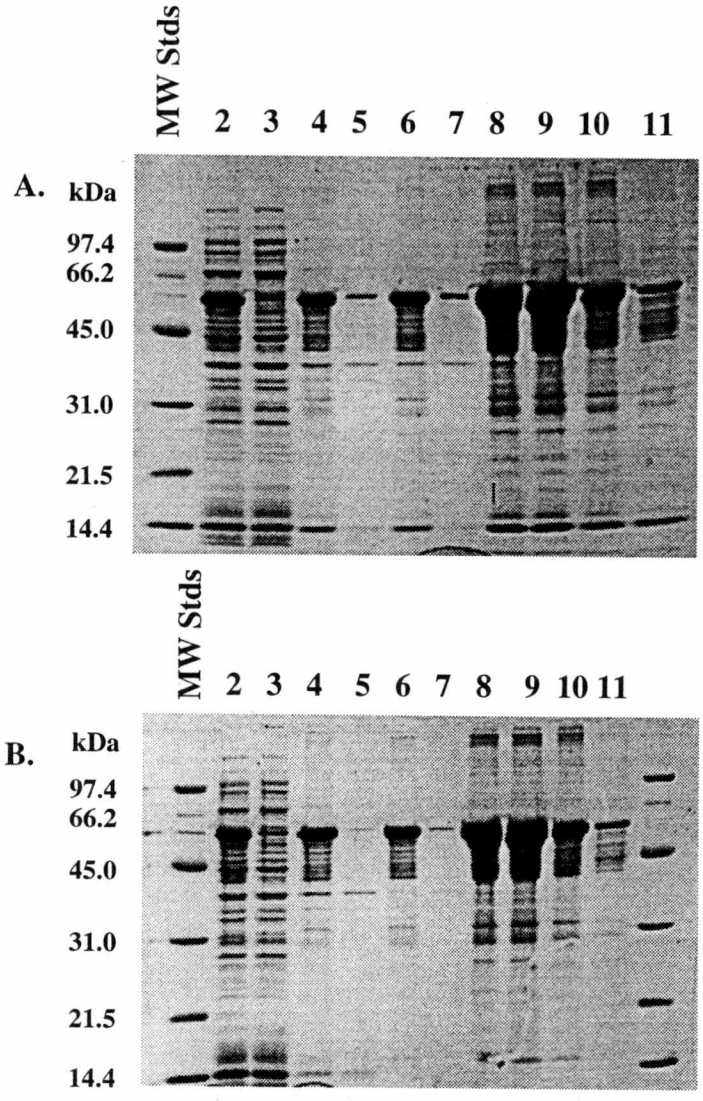


Fig. 3.12 : Purification of expressed hGABA-T.

The steps of purification of the expressed protein were followed using two different washing procedures described in the experimental procedures.

Rinsing of the insoluble protein was carried out in 50 mM TE buffer pH 8.0 containing 1mM ß-mercaptoethanol and 0.1% Triton X-100 (A) and 50 mM TE Buffer pH 8.0 containing 0.5% Triton X and 8% sucrose (B). Solubilization was carried out in 6M urea in lysis buffer.

Lane 2 - whole cell Lysate
3 - supernatant fraction 1
4 - pellet 1
5 - supernatant fraction 2
6 - pellet 2
Lane 7 - supernatant fraction 3
8 - pellet 3
9 - solubilized protein in 6M urea
10 - pellet 3 in 1M urea
11 - supernatant fraction in 1M urea

maintain reduced sulfhydryls. In the purification of GABA-T from mammalian tissues, it was found that although addition of PLP was not required at any step of the purification, an active enzyme with intact PLP was obtained (Churchich and Moses, 1981). The observations with the recombinant protein seem to suggest that PLP is not bound to the protein. Perhaps this could be a result of aggregation, which may mask the site for the modification, or alternatively, the rate of synthesis may be too fast to allow for the process to be carried out before aggregation.

Although conditions were eventually derived which yielded a soluble protein, no enzyme activity was detected by either the coupled assay system (Churchich and Moses, 1981) or the fluorescence technique (Beeler and Churchich, 1978), using sodium pyrophosphate buffer, pH 8.4, Tris-HCl buffer or potassium phosphate buffer, pH 8.0, as the assay buffer, with or without PLP. All of these buffers have been successfully used to assay for GABA-T; pyrophosphate buffer has been used for pig brain and liver GABA-T (Churchich and Moses, 1981), and recombinant pig brain GABA-T (Park *et al.*, 1993); Tris buffer has been used for recombinant rat brain GABA-T (Medina-Kauwe *et al.*, 1994); Tris or phosphate buffer is used in assays of purified human brain GABA-T (White and Sato, 1978), although this enzyme is inhibited by Tris to an extent of approximately 60%.

Since the solubilization was carried out on the crude enzyme preparation without prior purification, further purification of the enzyme was persued under denaturing conditions by affinity chromatography on the nickel column. Attempts were then made to refold the more highly purified enzyme to see whether catalytic activity could be recovered.

Affinity chromatography of expressed protein on His.Bind column

Results obtained following purification by affinity chromatography under denaturing conditions on the nickel column and monitored by SDS-PAGE and Western blot are shown in Figs. 3.13&3.14. The results indicated that the crude enzyme preparation, after the rinsing steps, was quite pure, and that most of the other proteins were removed at the washing step at low imidazole concentration (5 mM). The pooled, eluted protein was diluted 50% by addition of an equal volume of Tris buffer containing 6 M urea and 100 mM dithiothreitol (DTT) and 0.2 mM PLP to give a final concentration of 50 mM DTT and 0.1 mM PLP. The removal of urea was carried out by step-wise dialysis from 4 M to 2 M to 1 M urea. Finally, one half the volume was dialyzed in 50 mM Tris-EDTA buffer, pH 8.0, while the other half was dialyzed in 50 mM potassium phosphate buffer, pH 7.4. A soluble protein preparation was obtained for the sample dialyzed in Tris buffer, while partial precipitation was observed for the fraction dialyzed in phosphate buffer.

To investigate further the precipitation of the protein in phosphate buffer, equal amounts of the soluble enzyme were added to 1 ml of potassium phosphate or Tris buffer at different ionic strengths and different pHs between 7.4 and 8.5 and incubated at 25°C for 1 h. The samples were centrifuged and aliquots separated by SDS-PAGE and the bands visualized by silver staining and Western blot. The results obtained are shown in Figs. 3.15A&B, indicating that there was appreciable loss of protein from solution in the phosphate buffer particularly at lower ionic strength, compared to Tris buffer. Studies by Thatcher (1994), indicated that relative inclusion body solubility decreases in the following order: Guanidinium$^+$ >Li^+ >K^+ >Na^+ for cations and SCN^- >I^- >Cl^- for anions. Extrapolating this to the results obtained for the difference in

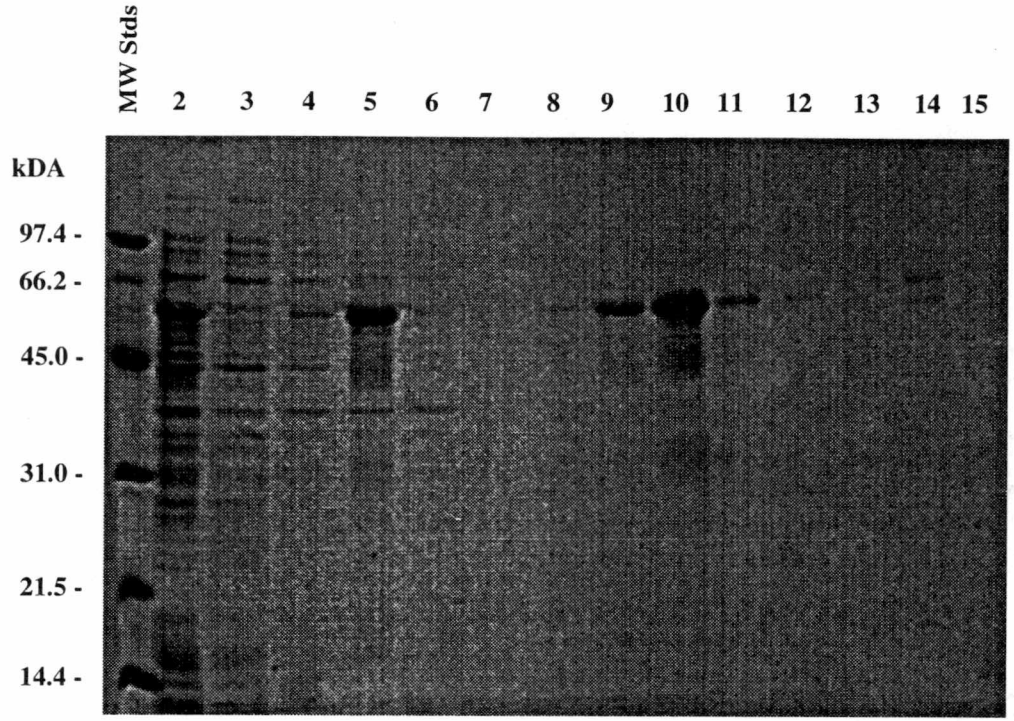


Fig. 3.13 : Affinity chromatography on His.Bind column followed by SDS-PAGE and Coomassie blue staining.

The recombinant protein was solubilized in Binding Buffer containing 6M Urea and elution carried out as described in the "experimental procedures".

Lane 2 - Whole cell extract
3 - First supernatant after lysis in 50mM Tris HCl / 2mM EDTA, pH 8.0
4 - Second supernatant after rinsing the precipitate in 50mM Tris HCl / 2mM EDTA, pH 8.0
5 - Aggregated protein solubilized in Binding Buffer containing 6M urea
6 - Flow-through on column
7 - Wash with Binding Buffer containing 6M urea
8 - Wash with above buffer containing 20 mM imidazole
9 -15 - Fractions collected with Elute buffer (300 mM imidazole)

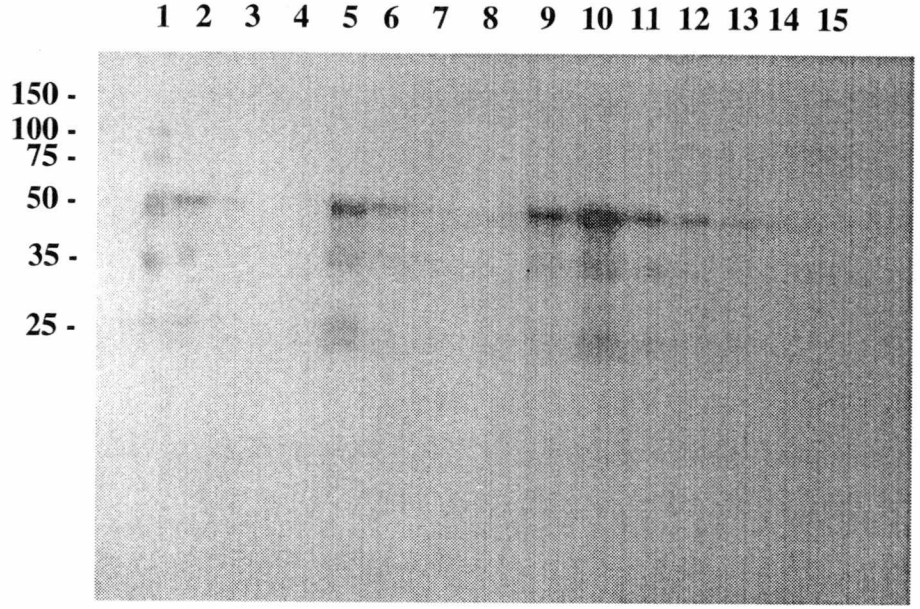


Fig. 3.14 : Western Blot Analysis used to follow purification of the recombinant GABA-T by affinity chromatography.

The solubilized protein in Binding Buffer containing 6M urea was applied on the column and eluted as described in the "experimental procedures".

Lane 1 - Perfect Protein Western Markers
2 - Whole cell extract
3 - First supernatant after lysis in 50mM Tris HCl / 2mM EDTA, pH 8.0
4 - Second supernatant after rinsing the precipitate in 50mM Tris HCl / 2mM EDTA, pH 8.0
5 - Aggregated protein solubilized in Binding Buffer containing 6M urea
6 - Flow-through on column
7 - Wash with Binding Buffer containing 6M urea
8 - Wash with above buffer containing 20 mM imidazole
9 -15 - Fractions collected with Elute buffer (300 mM imidazole)

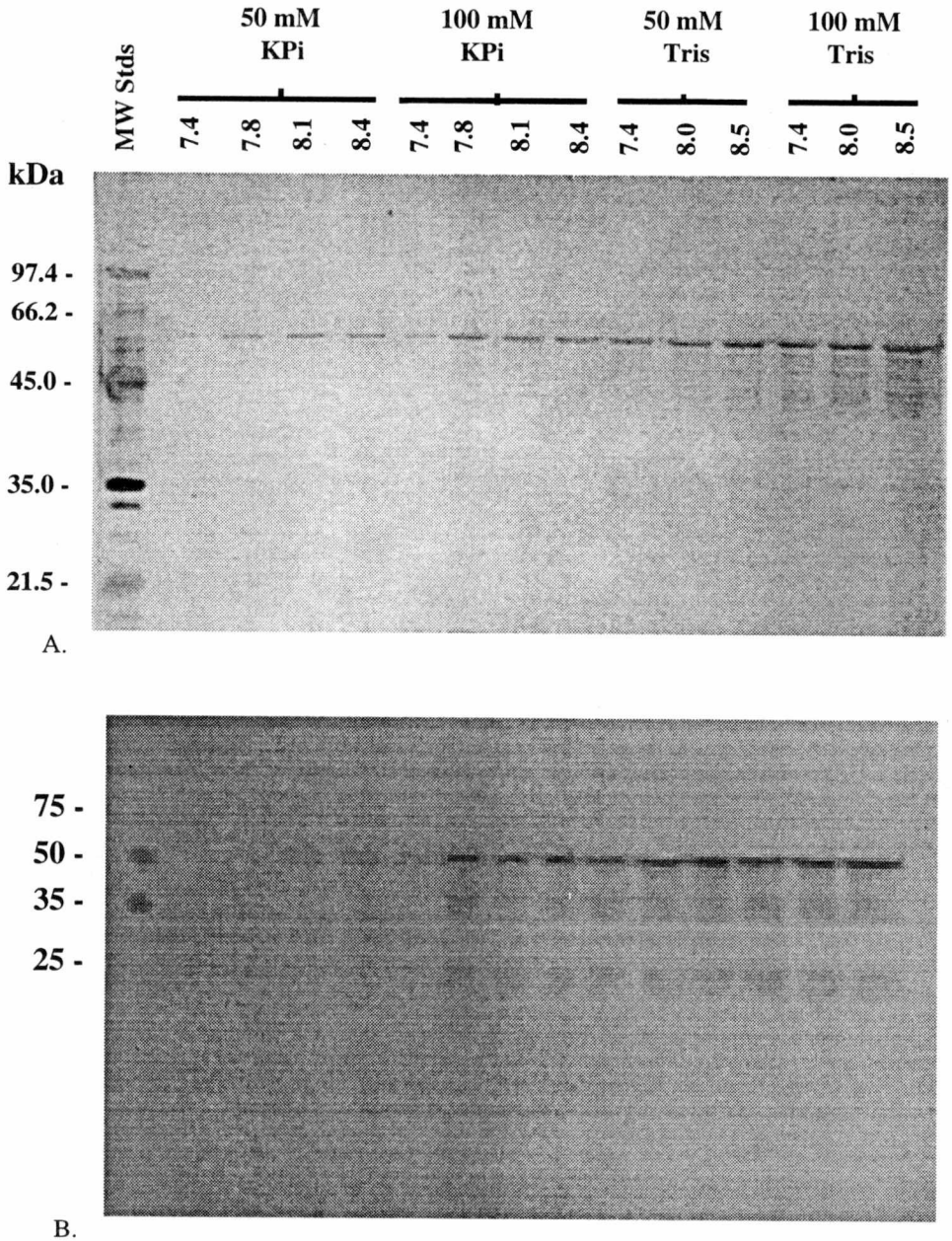


Fig. 3.15 : Solubility of the purified fusion protein in Tris and phosphate buffer. The enzyme preparation after His.Bind column chromotography was incubated in either Tris or potassium phosphate buffer at the different ionic strengths and pH's indicated. Aliquots from the incubation mixtures were monitored by SDS-PAGE and silver staining (A) or Western blot (B).

solubility in the two buffers, it may be inferred that the presence of either K^+ or PO_4^- could be affecting the solubility of the protein.

Although a soluble enzyme preparation was obtained in Tris buffer, enzyme activity was not detected. This may be the result of the N-terminal fusion portion of the expressed protein preventing the correct folding of the target enzyme. Therefore, thrombin digestion was carried out to remove the N-terminal oligohistidine portion of the fusion protein.

Thrombin digestion of soluble protein

Centrifugation of the sample after dilution of the solubilized protein to 2 M urea indicated that a little precipitation had occurred on dilution; however, the results of SDS-PAGE indicated the presence of the protein in the supernatant fraction. The procedure in the "experimental procedures" was followed to achieve conditions favorable for both the solubilization of the protein and thrombin activity under denaturing conditions. The protein has been shown to be soluble at 2 M urea, while 100% and 73% thrombin activities have been shown to be present after 2 h and 16 h incubation in 2 M urea, respectively, at 25°C (pET System Manual, Novagen). Results from SDS-PAGE indicated that there was some digestion of the protein, since a lower molecular weight band which was absent for the undigested sample appeared for the digested samples and the amount of cleavage increased with the increase in thrombin concentration. However, an increase of the incubation period did not result in any noticeable increase in the extent of the digestion. GABA-T activity was determined for the sample and was shown to be absent. Attempts to purify the digested protein on the nickel column in 2 M urea proved unsuccessful, as SDS-PAGE and Western blot

analysis used to follow the purification showed the same bands at all the stages of the purification.

For the initial subcloning into the pET30a vector, 23 out of the 27 amino acids forming the presequence had been deleted so that in effect the sequence of the mature form of the enzyme was subcloned. As presequences are normally required for targeting the protein into the mitochondrion and this process was redundant in the expression system it was thought its absence would cause no problem in the folding of the expressed protein. The advantages of using pET30a were that it has both the "His-Tag" and the "S-Tag" sequences which code for oligohistidine and the S-peptide, respectively, adjacent to the cloning site and these "Tags" provide for convenient purification and detection of the expressed protein. When attempts to obtain a functional protein failed with this vector, subcloning was carried out in pET12a which allowed the fusion of the insert to N-terminal signal sequence for potential periplasmic localization of the expressed protein. It was anticipated that such a system would remove the expressed protein from the cytoplasm into the periplasmic space, thus preventing its accumulation and intracellular degradation and/or misfolding.

Expression of hGABA-T subcloned into pET12a

The results obtained for induction time course for the expression of hGABA-T cDNA subcloned in pET 12a and transformed into BL21(DE3) cells are shown in Fig. 3.16. There was increased production of the protein with time after IPTG induction, but the protein was absent in the periplasmic fraction and present once again as an insoluble fraction. Unfortunately, for this vector system, there are no "Tags" to help in the detection of the expressed protein by Western blot. Therefore, the basis for evaluating whether the protein had been expressed was to compare the protein bands

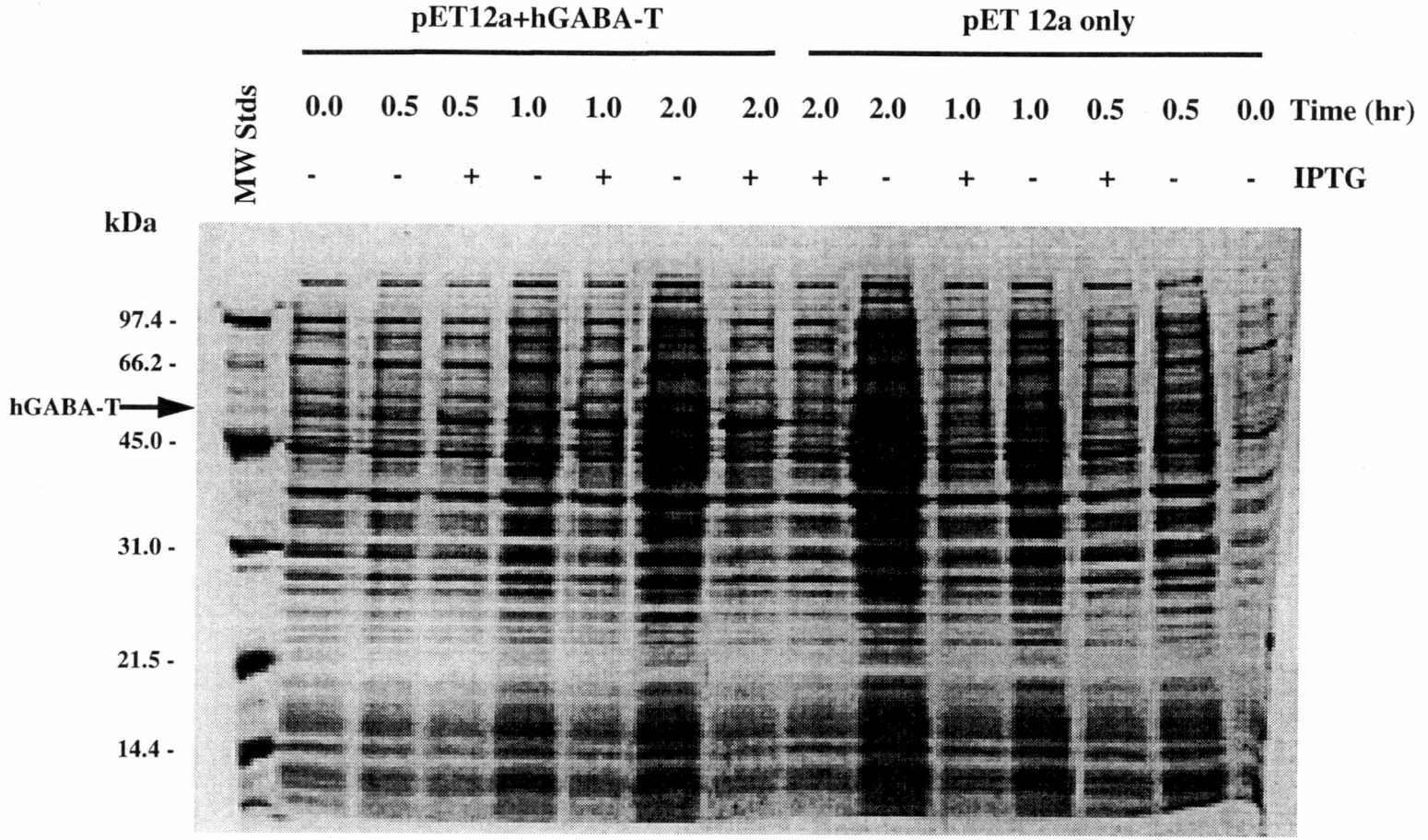


Fig. 3.16 : Time course of expression after IPTG induction of pET 12a +/- hGABA-T in BL21(DE3). Whole cell lysates were prepared for SDS-PAGE as described in the "experimental procedures" from cells grown in the presence/absence of IPTG.

observed upon Coomassie Blue staining of SDS gels from the lysates on cells containing the recombinant DNA to those with the vector DNA alone. From the results, only the cells with the recombinant DNA expressed a protein corresponding to the expected molecular weight of the expressed protein, and the protein was absent in either the uninduced cells or those transformed with the vector only.

The absence of the protein in the periplasmic fraction could be as a result of either the protein not being translocated, or aggregation after translocation due to an unfavorable environment in the periplasmic space. Possible interpretations of these results are that (i), the protein was being expressed at a rate far above the cells capability to keep it in a translocation-competent form for localization in the periplasmic space, thus resulting in its accumulation and leading to aggregation or (ii), that the translocated protein could not be processed at a rate fast enough to prevent aggregation as signal sequences have been shown to retard folding of precursor proteins *in vitro* (Park *et al.*, 1988; Laminet and Pluckthun, 1989). The possibility should also be considered that some of the protein was translocated to the periplasmic space, but was subsequently degraded. Since there were no antibodies available to detect the enzyme or its degraded form, the absence of the protein in the perisplasmic space should be interpreted with caution. Also, work by Gentz *et al.*, 1988 has provided some evidence for the presence of degraded proteins shortly after expression and secretion into the periplasm.

Once again, a soluble protein preparation was obtained after solubilization in 6 M urea followed by dialysis in Tris buffer, in the presence of DTT and PLP, but no enzyme activity could be detected. A possible explanation for this could be that the mature protein part could not fold properly into the native active form in the presence

of the presequence *in vitro*. It is generally understood that the presequences of authentic mitochondrial proteins are removed following import and presumably before folding and assembly (Hlodan and Hartl, 1994). The results on the recombinant hGABA-T also suggest that the precursor form has no catalytic activity or that the activity was too low to be detected. This observation disagrees with the previous observation made by Park *et al.*, (1993) in which the precursor pig brain enzyme, expressed in *E. coli*, showed enzyme activity. A final strategy was to co-express the recombinant gene with the GroEL/ES gene to evaluate whether the presence of the chaperones keeps the protein in a translocation-competent form for secretion into the periplasmic space.

Co-expression of hGABA-T and GroEL/ES

Fig. 3.17 shows the time course of expression of GABA-T by BL21(DE3) cells transformed with the recombinant pET12a/hGABA-T alone or together with the GroEL/ES plasmid DNA when monitored by SDS-PAGE and Coomassie blue staining on whole cell lysates. The cells containing both plasmids expressed mainly GroEL/ES, with the level of expression increasing with increase in the incubation period and not much expression of GABA-T (left half of Fig. 3.17), when compared to the cells containing the recombinant GABA-T gene only (right half of Fig. 3.17). Preparation of the periplasmic fractions for both cell cultures showed no protein band corresponding to the expressed protein when monitored by SDS-PAGE followed by Coomassie blue staining, indicating that co-expressing the recombinant gene and the GroEL/ES gene did not result in the translocation of the expressed protein into the periplasmic region. Complete lysis of the cells and SDS-PAGE of the supernatant fractions and pellets after centrifugation indicated the presence of the protein in the pellet, which could be solubilized in 6 M urea (Fig. 3.18). Thus the target protein was neither translocated nor

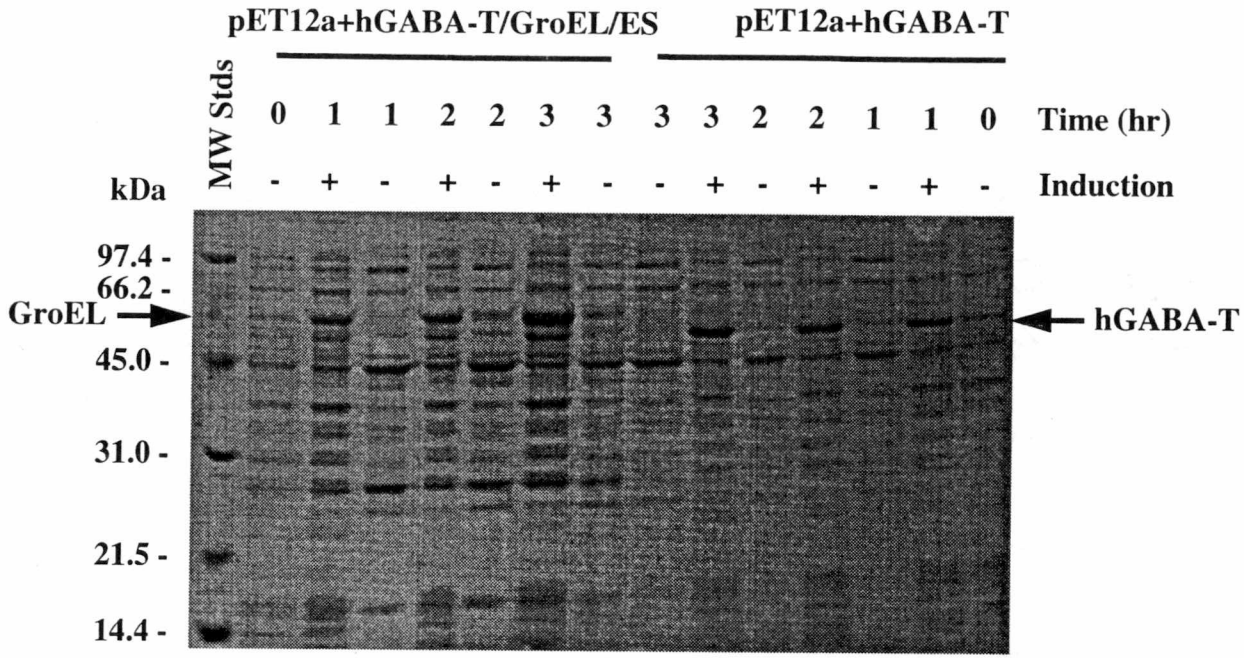


Fig. 3.17 : Induction time course of expressed hGABA-T in the presence or absence of GroEL/ES.

BL21 DE3 cells transformed with either plasmid GroEL/ES and pET12a+hGABA-T plasmids or pET12a+hGABA-T only were induced with IPTG and aliquots of cell cultures taken at the indicated time points and treated for SDS-PAGE and Coomassie blue staining as described in the "experimental procedures".

Fig 3.18 : SDS-PAGE following steps for the preparation of crude hGABA-T in the presence or absence of GroEL/ES.

Cells were harvested by centrifugation after a 3 h incubation at 25°C after IPTG induction. The cells were lysed by sonicating in 50 mM Tris buffer containing 2 mM EDTA and 1 mM β-mercaptoethanol. The lysate was centrifuged to pellet the insoluble fraction. The supernatant fraction (SN1) was decanted and the pellet resuspended in the lysis buffer, recentrifuged and the supernatant fraction (SN2) was decanted. The pellet was solubilized in the lysis buffer containing 6 M urea, 50 mM dithiothreitol and 0.1 mM PLP and incubated for 1 h to ensure complete solubilization. The urea was removed by dialyzing the samples against increasing concentrations of urea in the lysis buffer and finally in lysis buffer only. The soluble protein after dialysis is designated as dialysate in the figure. Aliquots of the various fractions were separated by SDS-PAGE and the protein bands stained using Coomassie blue stain.

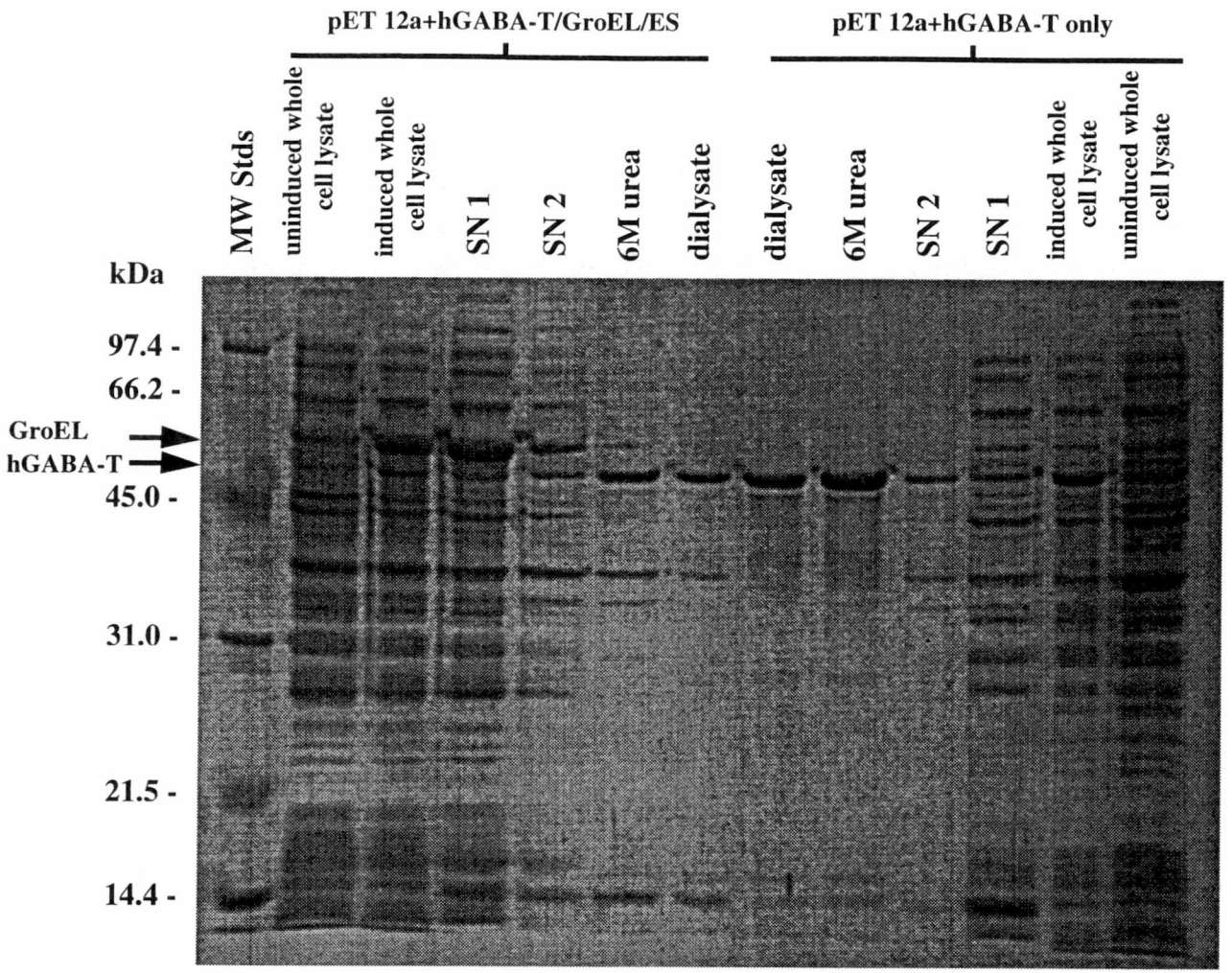


Fig. 3.18 : SDS-PAGE following steps for the preparation of crude hGABA-T expressed in the presence or absence of GroEL/ES.

SN1 - supernatant fraction 1 SN2 - supernatant fraction 2 6 M urea - solubilized fraction in 6 M urea

folded correctly to give a soluble protein in the presence of the chaperones. Once again the insoluble fraction was solubilized in urea and dialyzed as described previously to obtain the soluble protein which showed no enzyme activity.

Molecular chaperones have been shown to be involved in ensuring the fate of protein *in vivo* such as its folding, assembly or transport into a particular cellular compartment (Hendrick and Hartl, 1993). GroEL for example, has been shown to interact with many monomeric and multi-subunit proteins during their folding (Cheng *et al.*, 1989; Ostermann *et al.*, 1989; Martin, *et al.*, 1991) and to be involved in sorting certain precursor proteins to both the bacterial periplasm (Bochkareva et al., 1988) and within the mitochondria into the compartments of the organelle (Koll *et al.*, 1992). It has been shown that GroEL interacts with proteins in partially unfolded states which retain some amount of secondary structure (Langer *et al.*, 1992). A success rate of about 25-30% has been observed for the production of soluble protein when target proteins were co-expressed with the chaperones (Gatenby, personal communication). Recently, Amrein *et al.*, (1995) showed that large quantities of recombinant human protein-tyrosine kinase were produced when the recombinant gene was expressed in an *E. coli* system over-producing the bacterial chaperones GroEL and GroES. Since the chaperones did not have a positive impact in hGABA-T expression, the unfolded intermediates may be more prone to aggregation than to follow the pathway leading to the correctly folded native protein.

SUMMARY AND FUTURE STUDIES

Physical studies of GABA-T indicated that the protein dissociated at pH 5.0 into the monomeric species which retained secondary structure and showed no catalytic activity. The dissociation was reversible and was characterized by a relaxation time of 18 ms; however the rate of association was too fast to be monitored. The residues at the interface of the subunits and the type of interactions involved in dimerization are unknown. Future studies involving x-ray crystallography and mutagenesis of some residues that might be indicated at the interface from structural studies will be helpful in elucidating the type of interaction present at the interface.

The cDNA for human brain GABA-T has been isolated and sequenced. The open reading frame consists of 1503 bp coding for 500 amino acids with the mature enzyme consisting of 473 amino acids, with the remaining N-terminal 27 residues assigned to the presequence. Comparison with known sequences from other sources revealed high sequence identity (~ 95 %) among brain and liver enzymes from rat, pig and human. Sequence identity of 42 % and 28 % were observed for yeast and bacterial GABA-T, respectively.

Northern blot analysis detected a major transcript of approximately 5.7 kb which was present in different amounts in the different tissues. Detection levels of GABA-T mRNAs were in the following (in decreasing order): liver > pancreas > brain > kidney > heart > placenta and absent in the lung and skeletal muscle. It would be interesting to explore further the role of GABA-T in these tissues. Initial attempts to map the gene on the human chromosome were unsuccessful, and this could be pursued

in future experiments for the detection of any chromosomal abnormalities in people prone to conditions such as epileptic seizures.

The gene for human brain GABA-T has been successfully cloned into the expression vectors, pET30a and pET12a, for expression in *E. coli* under strict control of the T7 promoter. The expressed protein was insoluble, and manipulations such as growth of cells at lower temperatures, higher salt concentrations or reduced growth periods did not result in the production of a soluble protein. Co-expression of GABA-T gene and the gene for the co-chaperones, GroEL/ES, was likewise ineffective in producing a soluble protein. A soluble protein was obtained by dissolving the aggregated protein in 6 M urea in the presence of a high concentration of the reducing agent, dithiothreitol, and PLP, followed by stepwise dialysis in Tris buffer containing decreasing concentrations of urea. No catalytic activity was detected for the refolded protein.

The first choice of expression systems for the current study was the *E. coli* expression system. The reasons for choosing this system were that a number of eukaryotic genes have been successfully expressed in *E. coli*, and that GABA-T does not require post-translational modification or disulfide bond formation. Therefore, problems were not anticipated with the protein expressed in the reducing environment present in *E. coli*, a system where modification is usually not accomplished for expressed foreign proteins. Since an insoluble protein was obtained with the system used, it is proposed that other strategies be tried to identify effective solubilization reagents and protocols, as different insoluble proteins have been shown to require different procedures which can only be determined empirically.

Secondly, *E. coli* expression systems other than the pET expression system employed could be used to determine whether better results may be obtained. This is proposed as an alternative since a large amount of degraded protein was observed with the system used, and this could have contributed to the aggregation of the protein. Finally, although the mammalian expression system has been shown to require a great deal of time and effort to obtain stable cell lines (Goeddel, 1991), it will be worth trying in the event that expression in all bacterial systems fails to produce a soluble protein and the solubilization protocols developed also fail to yield soluble and active protein. The over-expression of soluble and active GABA-T should facilitate future studies on testing and developing newer drugs with anti-convulsant action at inhibiting GABA-T activity in humans.

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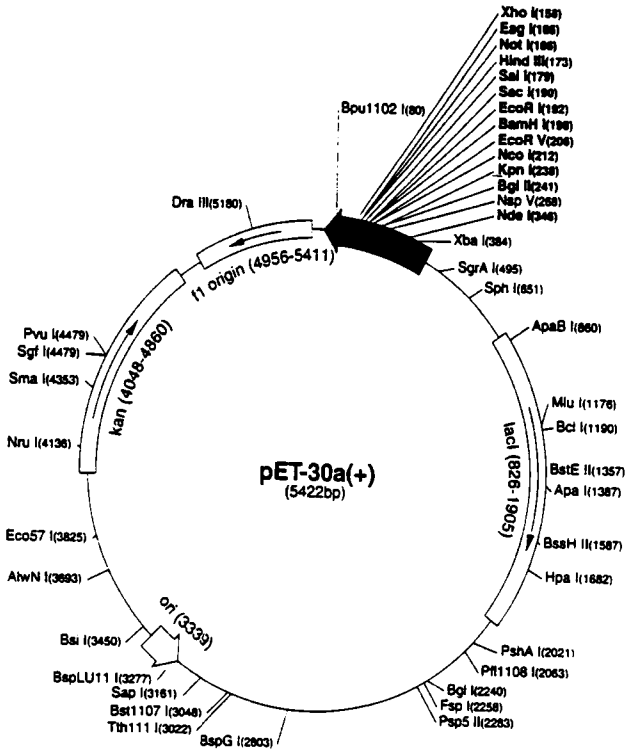
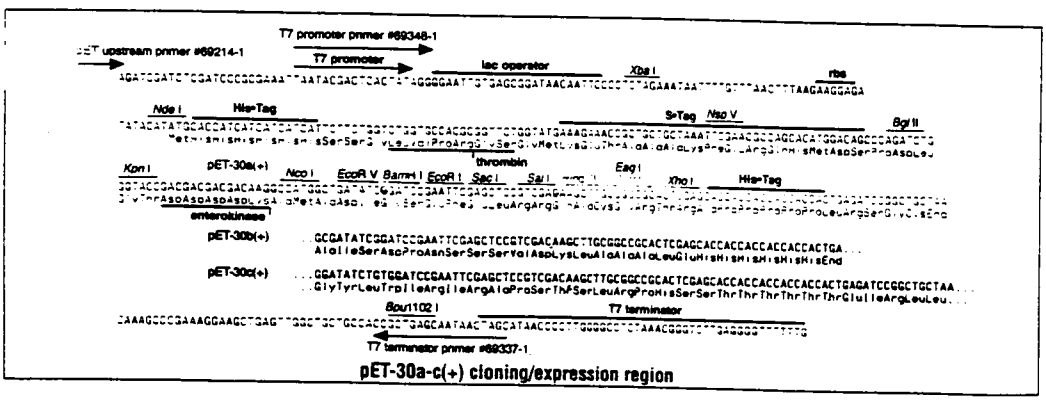
APPENDICES

pET-30a(+) sequence landmarks	
T7 promoter	419-435
T7 transcription start	418
His•Tag coding sequence	327-344
S•Tag coding sequence	249-293
Multiple cloning sites	
(*Nco* I - *Xho* I)	158-217
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	826-1905
pBR322 origin	3339
Kan coding sequence	4048-4860
f1 origin	4956-5411

The maps for pET-30b(+) and pET-30c(+) are the same as pET-30a(+) (shown) with the following exceptions: pET-30b(+) is a 5421bp plasmid: subtract 1bp from each site beyond *Bam*H I at 198. pET-30c(+) is a 5423bp plasmid: add 1bp to each site beyond *Bam*H I at 198.

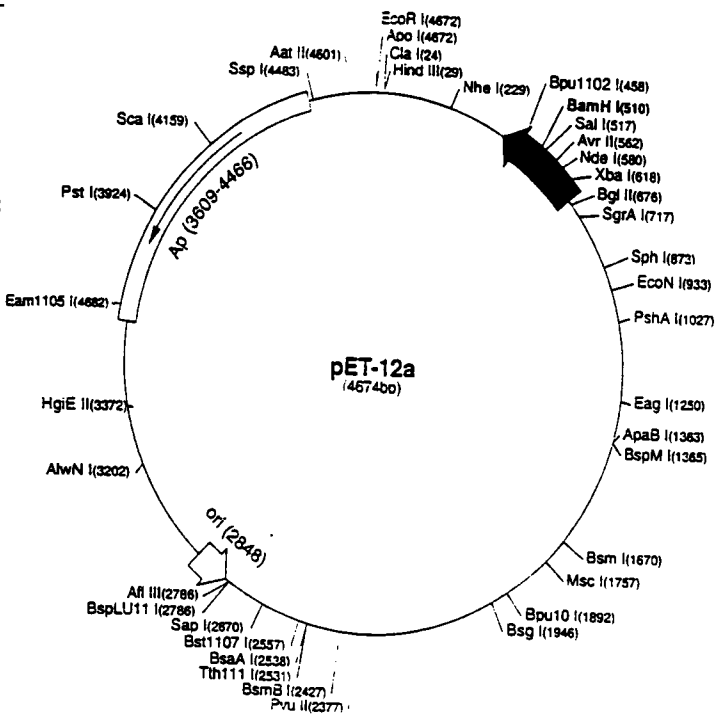


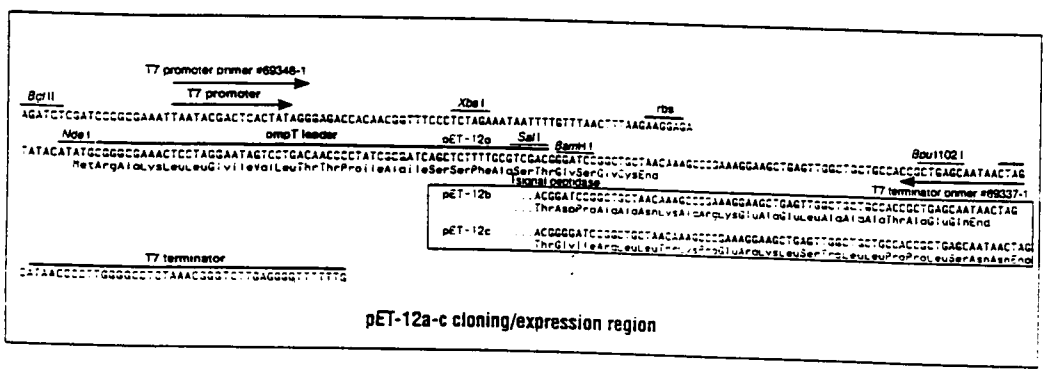


A1.1: Vector Map for pET30a (Novagen).

pET-12a sequence landmarks	
T7 promoter	645-661
T7 transcription start	644
ompT coding sequence	516-578
T7 terminator	404-450
pBR322 origin	2848
bla coding sequence	3609-4466

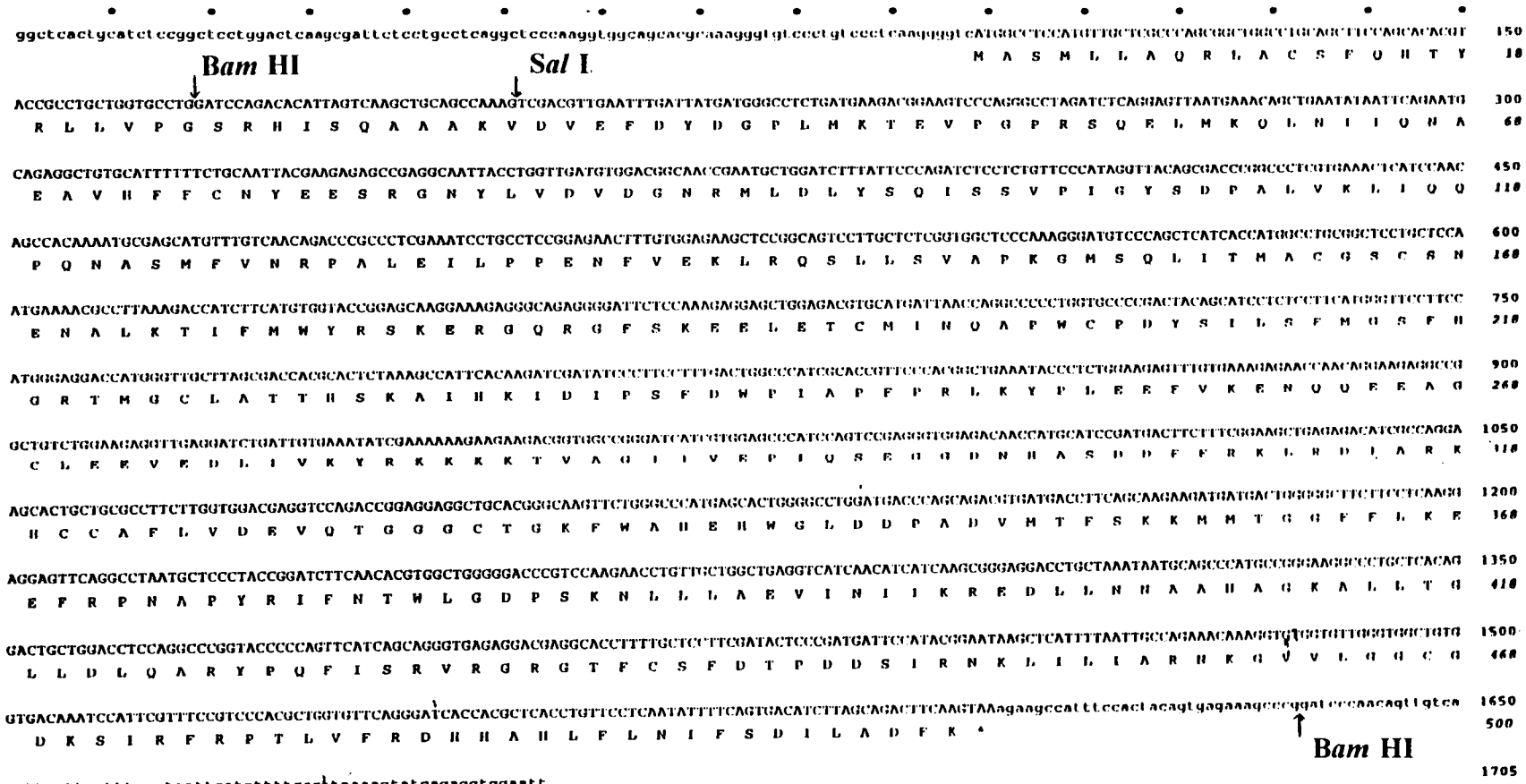
The maps for pET-12b and pET-12c are the same as pET-12a (shown) with the following exceptions: pET-12b is a 4673bp plasmid; subtract 1bp from each site beyond *BamH* I at 510. pET-12c is a 4675bp plasmid: add 1bp to each site beyond *BamH* I at 510.





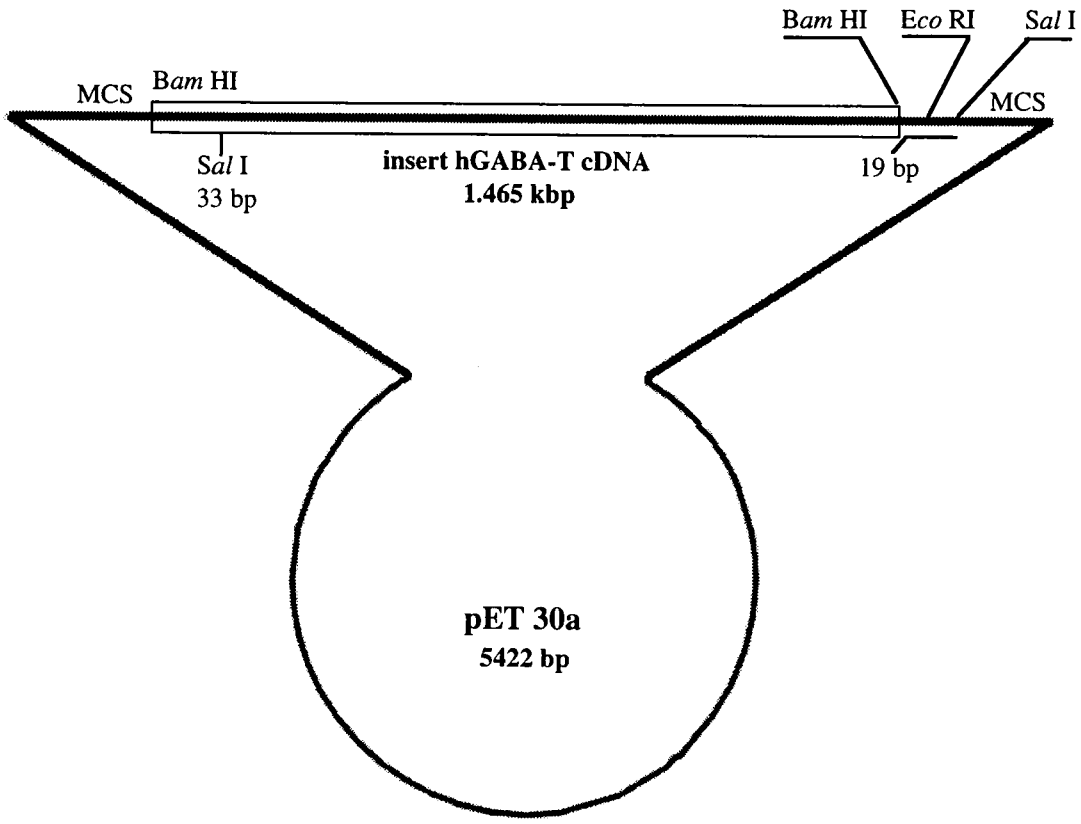
pET-12a-c cloning/expression region

A1.2: Vector Map for pET12a (Novagen).

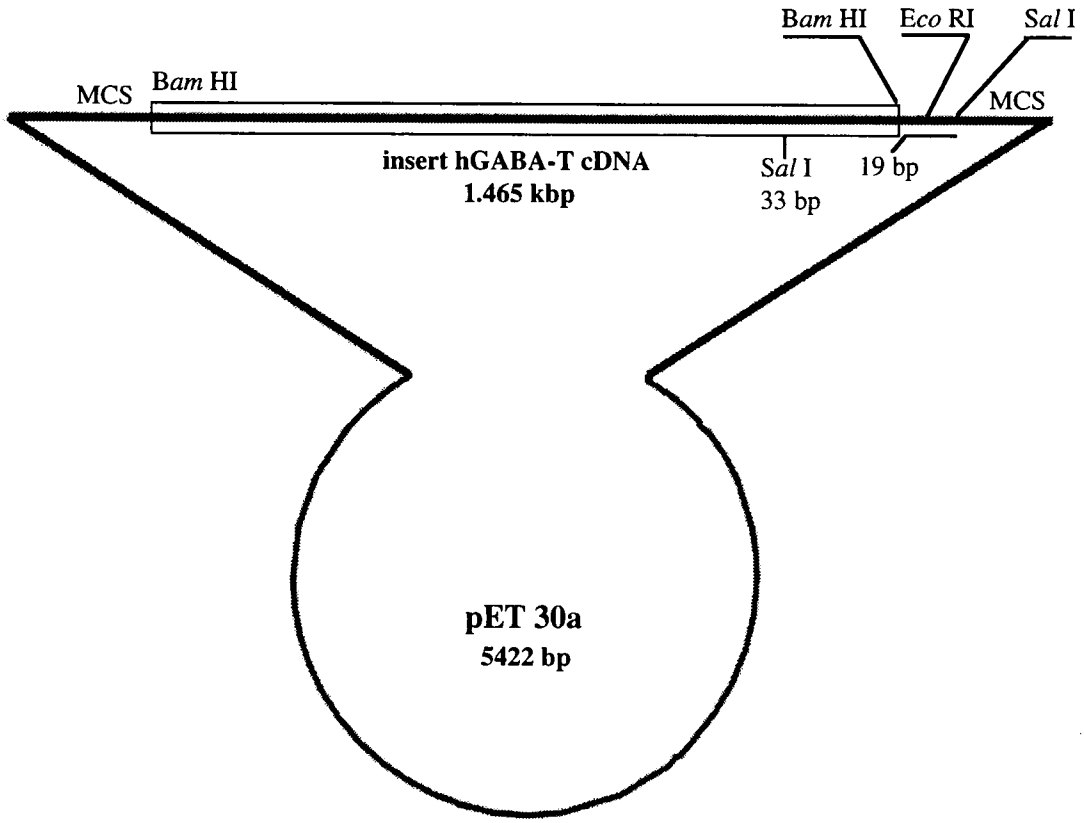


A2.1: Nucleotide and deduced amino acid sequences of hGABA-T showing restriction sites for B*am* HI and S*al* I.

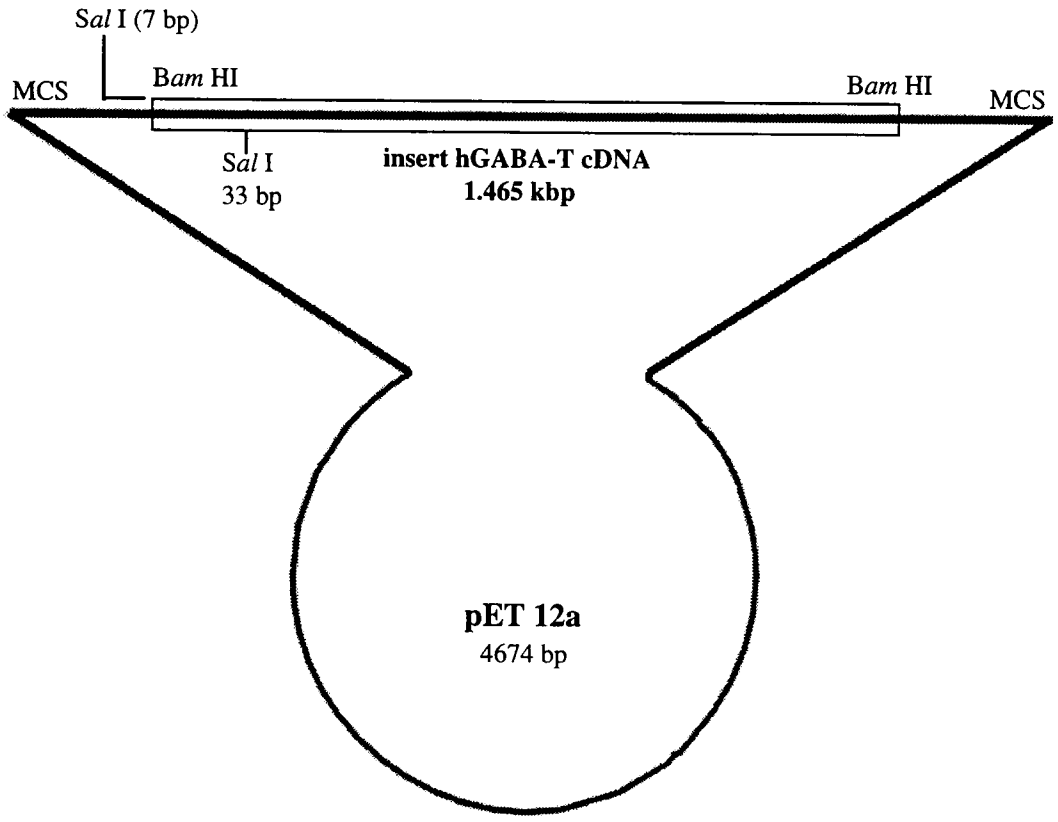
The predictions were made using the GCG program. The numbering of amino acid residues is shown in italics.



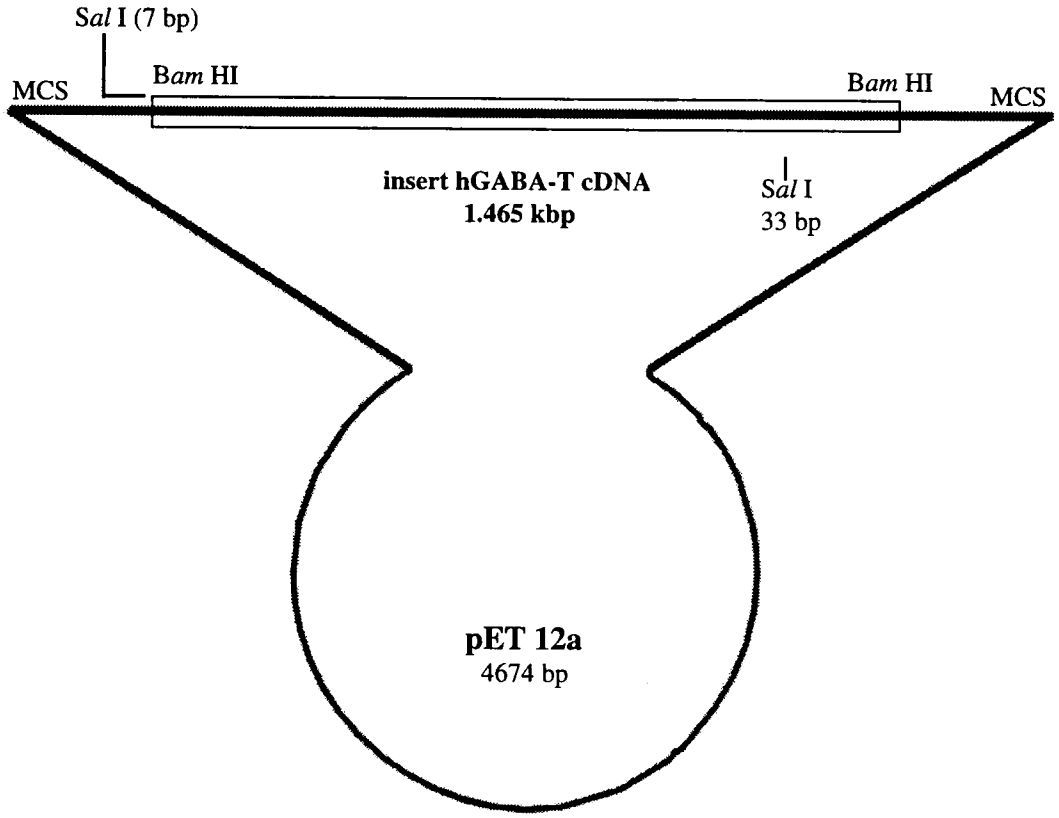
A 3.1 : Diagrammatic representation of insertion of hGABA-T cDNA into pET 30a in the correct orientation



A 3.2 : Diagrammatic representation of insertion of hGABA-T cDNA into pET 30a in the wrong orientation



A 3.3 : Diagrammatic representation of insertion of hGABA-T cDNA into pET 12a in the correct orientation



A 3.4 : Diagrammatic representation of insertion of hGABA-T cDNA into pET 12a in the wrong orientation

VITA

Yaa Difie Osei was born in Bekwai-Ashanti, Ghana, on June 1, 1949. She completed her High School Education at Wesley Girls' High School, Cape Coast, Ghana in 1969 and entered the University of Ghana, Legon, in October of the same year. She graduated with a Bachelor of Science (General) degree in Biochemistry, Nutrition and Food Science in July, 1972 and a Bachelor of Science (Honors) degree in Biochemistry, in 1973. She was awarded the University of Ghana Graduate Scholarship for her Master's degree program in the same University and obtained a Master of Science degree in Biochemistry in November, 1977. During her undergraduate program, she received the Waddel Prize (a University of Ghana annual prize for the best undergraduate science student), for 1970/71 and 1971/72 academic years. In the summer of 1972, Yaa received a fellowship from the Association for the Exchange of International Students for Technical Experience to Norway, where she worked in a food control laboratory.

Yaa worked as a Research/Teaching Assistant at the Department of Biochemistry, for a year on National Service and was employed initially, as an Assistant Research Officer in 1978 and promoted to a Research Officer in 1980 at the Food Research Institute, Accra, Ghana. In January 1979, she was awarded a Dutch Government Fellowship through the Netherlands Universities Foundation for International Cooperation for a diploma course in Nutrition and Food Science and obtained a Diploma with distinction in June, 1979.

In October 1980, Yaa accepted a position as lecturer at the Department of Biochemistry, University of Ghana where she taught mainly undergraduate courses to

students in the faculties of Science and Agriculture. Her research projects in Ghana centered mainly on medicinal plants and Nutritional disorders.

In the fall of 1991, Yaa received a fellowship from AFGRAD program through the African-American Institute (AAI) for her Ph. D. program at the University of Tennessee, Knoxville. During her first year of graduate studies, she enjoyed a tuition waiver from the Graduate School, UTK and subsequently Teaching Assistantship with tuition waiver from the Department of Biochemistry, UTK. She was one of the three recipients at the Department for the Science Alliance Award in 1994. Yaa was on study-leave from the University of Ghana and will return to her post after the completion of her Ph. D program in December, 1995.

Yaa is a member of the following professional Associations:

The Ghana Biochemical Soceity

The Ghana Science Association

The Association of Women in Science and Technology (Ghana)

Third World Organization for Women in Science.

Her research publications at UTK include the following:

Osei, Y. D. and Churchich, J. E. (1995) Screening and sequence determination of a cDNA encoding the human brain 4-aminobutyrate aminotransferase. Gene **155**, 185-187.

Pineda, T., **Osei, Y. D.** and Churchich, J. E. (1995) Characterization of monomeric 4-aminobutyrate aminotransferase at low pH. Eur. J. Biochem. **228**, 683-688.

Park, J., **Osei, Y. D.** and Churchich, J. E. (1993) Isolation and Characterization of Recombinant Mitochondrial 4-Aminobutyrate Aminotransferase. J. Biol. Chem. **268**, 7636-7639.

She participated in a workshop on "Women in Leadership" organized at Southern University, Baton Rouge, Louisiana , by AAI from December, 16-21,1992. She also presented a poster at the Annual Meeting of The American Society for Biochemistry and Molecular Biology at Washington D.C., May 21-25, 1994.