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Molecular Identification of the Fish 4-Aminobutyrate Aminotransferase from Flounder, *Paralichthys olivaceus*

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4-Aminobutyrate aminotransferase plays an essential role in the 4-aminobutyric acid shunt, converting 4-aminobutyrate to succinic semialdehyde. We isolated and sequenced a fish cDNA fragment that encodes 4-aminobutyrate aminotransferase. A brain cDNA library from flounder (*Paralichthys olivaceus*) was constructed using the ZAP-III XR vector and screened for the fish 4-aminobutyrate aminotransferase gene using a probe derived from the conserved sequences of known mammalian 4-aminobutyrate aminotransferases. A partial cDNA for 4 -aminobutyrate aminotransferase was cloned and found to be 700 bp in length corresponding to 66 amino acids. Nucleotide sequence of the clone was aligned with NCBI (National Center for Biotechnology Information) DNA sequence data base. The result showed high sequence identity with previously reported mammalian 4-aminobutyrate aminotransferases. The transcriptional level of flounder 4-aminobutyrate aminotransferase was detected with the presence of mRNA at different flounder tissues by reverse transcription-polymerase chain reaction (RT-PCR). The expression of flounder 4-aminobutyrate aminotransferase was also tested and detected from the flounder tissues of the brain, liver, kidney and pancreas.

Key words: Flounder, *Paralichthys olivaceus*, 4-Aminobutyrate aminotransferase, Molecular cloning, Transcription, Expression

Introduction

4-Aminobutyrate (GABA) is a major inhibitory neurotransmitter in the mammalian central nervous system and is produced by decarboxylation of glutamic acid through the action of cytosolic enzyme, glutamate decarboxylase, and converted into succinic semialdehyde by mitochondrial enzyme, 4-aminobutyrate aminotransferase (McCormick, 1989). GABA levels are of crucial importance in controlling convulsion in humans and animal models, and there is a correlation between the deranging GABA metabolism and the appearance of various system disorders (De Biase et al., 1995). 4-aminobutyrate aminotransferase is not restricted to the central nervous system but is also found in several peripheral tissues, such as liver, kidney, pancreas, and blood platelets. 4-aminobutyrate aminotransferase has been purified from brain and liver of several mammals including mouse, rat, rabbit, pig, and human (Beeler and Churchich, 1978; Bloch-Tardy et al., 1974; Buzenet et al., 1978; Schousboe et al., 1973; 1977). There were several reports on amino acid sequences of mammalian 4-amiobutyrate aminotransferase, deduced from cDNA sequences from pig (Kim et al., 1991; Kwon et al., 1992), human (Park et al., 1993; Osei and Churchich, 1995), and rat (Medina-Kauwe et al., 1994).

Biochemical and molecular biological studies on the mammalian 4-aminobutyrate aminotransferase were performed to obtain the structural and functional characteristics of that enzyme (Kim and Churchich, 1989; 1991; Lee et al., 1996; Kim et al., 1995; 1997; Sung et al., 1999) but there have been no such studies on the fish 4-aminobutyrate aminotransferases. Therefore, investigations on the 4-aminobutyrate aminotransferase derived from fish species are important for understanding the fish central nervous system and their crucial metabolism. In this study, we describe

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the molecular cloning of 4-aminobutyrate aminotransferase from flounder (*Paralichthys olivaceus*) and the sequence analysis of the partially cloned cDNA with other mammalian 4-aminobutyrate-aminotransferases.

Materials and methods

Materials

The *E. coli* strain BL21 (DE3) (*F⁻ompT hsdS$_B$* ($r_B^-m_B^-$) *gal dcm* (*DE3*)) was used in this study. The *E. coli* strain XL1-Blue MRF′ (F′::*Tn10 proA*$^+$ *B*$^+$ *lacI*^{q} *Δ*(*lacZ*) *M15/recA1 endA1 gyrA96*(*Nal*^{r}) *thi hsdR17*($r_K^-m_K^+$) *supE44 relA1 lac*) was used for library titration, transformation, and color selection. The primers used for this study are summarized in Table 1 and synthesized from Genotech (Korea). Restriction enzymes, reverse transcriptase, and RNasin® Ribonuclease inhibitor were purchased from Promega (U.S.A.). T4 DNA ligase and *Taq* DNA polymerase were obtained from United States Biochemicals Corp. (U.S.A.) and TAKARA (Japan), respectively. ABI PRISM™ dye terminator Cycle sequencing ready reaction kit was purchased from PERKIN ELMER Inc. (U.S.A.).

Goat anti-mouse IgG alkaline phosphatase conjugated antibody was purchased from Serotech (U.S.A.). Digitogenin (DIG)-labelling and detection kit were purchased from Boeringer Mannheim (Germany). NBT/BCIP for alkaline phosphatase color developing solutions were purchased from Bio-Rad (U.S.A.). Other chemicals were purchased from Sigma (U.S.A.), Difco (Madison, U.S.A.), and Fluka (U.S.A.).

Total RNA isolation and construction of cDNA library

The preparation of total RNA from flounder tissues of brain, liver, kidney and pancreas was performed with total RNA isolation kit (Promega, U.S.A.). Complementary DNA library was constructed as described in the manufacturer's instruction (Stratagene, U.S.A.). Messenger RNA was isolated from flounder brain tissue using oligo (dT) cellulose. Flounder cDNA was synthesized with MMLV-reverse transcriptase using a linker-primer and second-strand cDNA was synthesized with DNA polymerase and RNase H. Synthesized cDNA was blunt-ended at the termini, ligated to the *EcoR* I adaptors, and digested with *Xho* I. And then, cDNA was ligated with ZAP Express vector. This ligation mixtures were packaged with the Gigapack® III Gold packaging extract (Stratagene, U.S.A.).

Screening of 4-aminobutyrate aminotransferase from flounder cDNA library

Conserved sequences of mammalian 4-aminobutyrate aminotransferases were determined using nucleotide and protein sequence database, and oligonucleotide probe (Table 1) for screening was synthesized and labeled with DIG-oligonucleotide 3′-end labeling kit (Boehringer Mannheim). Positive plaques were screened with above probe and further confirmed by the second screening.

Table 1. Oligonucleotide primers used for probe preparation

Primer	Direction	Sequence
fGT-F	forward	5′-AC(C/T)CATGGGTTGCTTAGCGAC-3′
fGT-R	reverse	5′-C(A/C)GTCATCATCTTCTTCCTG-3′

In vivo excision and DNA sequencing

Positive plaques were recovered from the second screening and the phagemid containing the insert was excised according to the manufacturer's instructions (Stratagene). Sequencing of the excised phagemid was performed by the method described in (Kim and Richardson, 1993; 1994) and ABI 310 Genetic Analyzer according to the manufacturers instructions (Perkin Elmer).

Reverse transcription-polymerase chain reaction (RT-PCR)

In order to perform reverse transcription polymerase chain reaction, total RNA was isolated from the flounder tissues of the brain, liver, kidney and pancreas as described in (Cho et al., 1999). Twenty microliters of the reaction products were separated by electrophoresis through 1.5% agarose gels and stained with Etidium bromide.

Other methods

Immunoblot analysis of 4-aminobutyrate aminotransferase in brain tissue was performed by using mouse polyclonal antibodies prepared against purified pig brain 4-aminobutyrate aminotransferase. Procedures for transfer of proteins from 12% SDS-polyacrylamide gels to nitrocellulose blots have been

described (Kim et al., 1997). SDS-PAGE was performed by the method of Laemmli (1970). Southern blot analysis was performed with brain cDNA fragment encoding 4-aminobutyrate aminotransferase. DNA was separated on a 1.5% agarose gel, transferred to nylon paper, and hybridized with DIG-labeled DNA fragment as a probe.

Results and Discussion

There were much informations on the enzymatic and structural characteristics on the 4-aminobutyrate aminotransferase in several mammals including pig, rat and human well are documented in recent years but little information available in fish (Sung and Kim, 2000). In the present study, we have identified of the fish 4-aminobutyrate aminotransferase showing that the fish also has the characteristics of the central nerve system and energetic metabolic process mediated by 4-aminobutyrate (GABA) like mammalian systems. However, there are no direct studies on the fish system of the shunt on the basis of 4-aminobutyrate aminotransferase. Therefore, investigations on the 4-aminobutyrate aminotransferase derived from fish species are important for understanding the critical roles of 4-aminobutyrate aminotransferase on the central nerve system and metabolic mechanisms. We have described the molecular identification of 4-aminobutyrate aminotransferase from flounder (*Paralichthys olivaceus*) and the sequence analysis of the partially cloned cDNA with other mammalian 4-aminobutyrate aminotransferases.

Detection of flounder 4-aminobutyrate aminotransferase

In order to detect the presence of fish 4-aminobutyrate aminotransferase from flounder, Western blot analysis was performed with polyclonal antibodies raised against mouse BALB/c strains by using purified pig brain 4-aminobutyrate aminotransferase. The extract from flounder brain tissues was separated on a SDS-PAGE and whole proteins were electrophoretically transferred from SDS-PAGE gel to nitrocellulose membrane, probed with polyclonal antibodies. As shown in Figure 1, 4-aminobutyrate aminotransferase was detected from flounder brain extract, showing that flounder also has the identical enzymatic system which is involved in GABA metabolism like mammalian system.

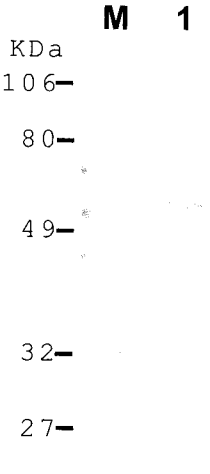

Fig. 1. Western blot analysis of flounder 4-aminobutyrate aminotransferase.
The extract from flounder brain tissues was electrophoretically transferred from SDS-PAGE gel to nitrocellulose membrane, probed with mouse ascites fluid against pig brain 4-aminobutyrate aminotransferase, and incubated with alkaline phosphatase coupled to goat antibody against mouse IgG. M, prestained SDS-PAGE standards, low range molecular weights (Bio-Rad); lane 1, flounder brain extract.

Cloning and sequencing

A flounder brain cDNA library was screened using a probe derived from the conserved domain of mammalian 4-aminobutyrate aminotransferase cDNA. A positive clone with a 700 bp insert was isolated and sequenced. The sequence of the cloned cDNA was aligned with NCBI nucleotide sequence data base using a Blast program and showed high sequence identity with mammalian aminotransferase, which indicates that the cloned DNA fragment is a gene for 4-aminobutyrate aminotransferase. Its nucleotide sequence was determined and found to be 700 bp in length corresponding to 66 amino acids (Figure 2). The nucleotide sequence of a partial clone from flounder shares 99% and 90% sequence homology with its sequence of pig and human of 4-aminobutyrate aminotransferase, respectively. As shown in Figure 3, the structural comparisons of the deduced amino acids sequences of flounder 4-aminobutyrate aminotransferase were conducted with the amino acids sequences of human (Osei and Churchich,

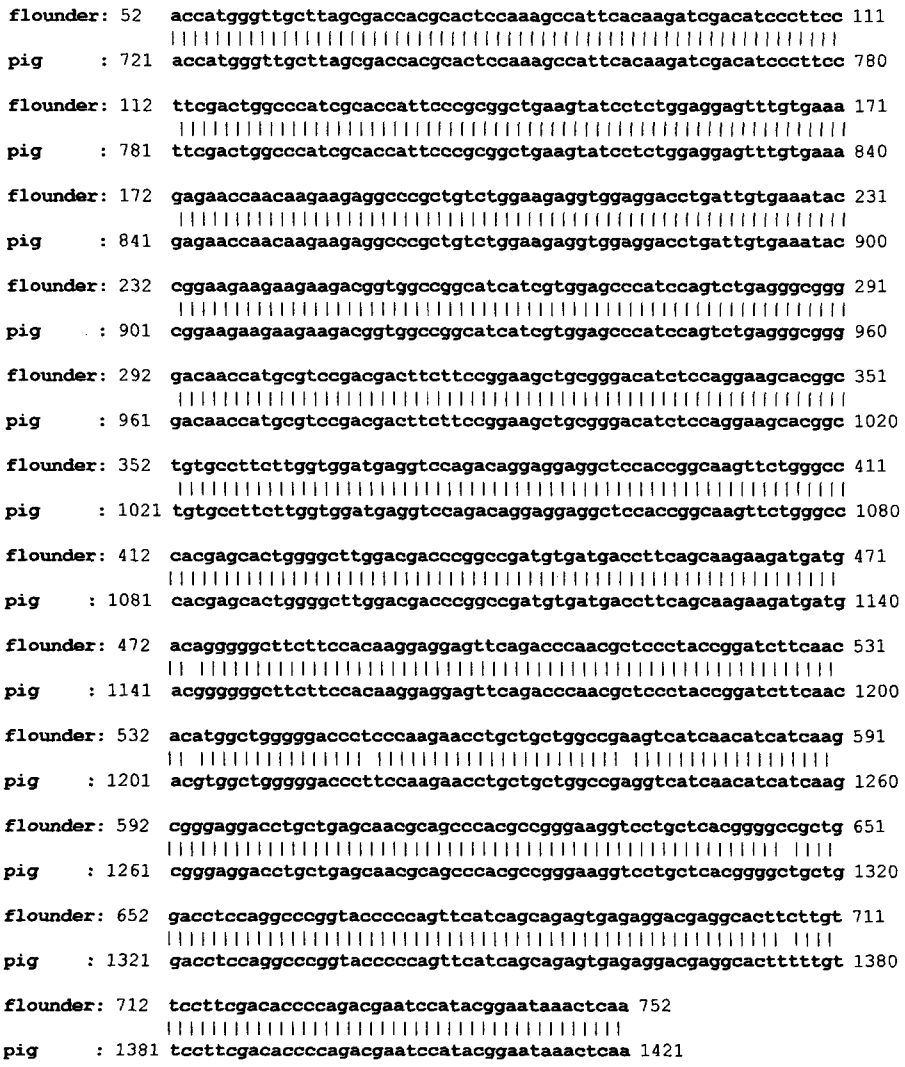

Fig. 2. DNA sequence alignment of a partially cloned DNA fragment of flounder 4-aminobutyrate aminotransferase with the sequence of pig brain 4-aminobutyrate aminotransferase.

1995), pig (Kwon et al., 1992), and rat (Tillakaratne et al., 1995) using Clustal Multiple Alignment Program (Higgins and Sharp, 1989). As indicated in Figure 3, the box designates a putative PLP binding site which is well conserved among the species. The sequence homology is 98% for pig, 96% for human, and 92% for rat. Sequence comparisons of the nucleotides and the amino acids of 4-aminobutyrate aminotransferase among inter-species were summarized in Table 2.

Table 2. Sequence comparisons of nucleotide and amino acid sequences of flounder 4-aminobutyrate aminotransferase with human, pig, and rat

Species	Sequence identity (nucleotide/amino acids, %)
	Flounder
Pig	99/98
Human	90/96
Rat	87/92

Tissue distribution of flounder 4-aminobutyrate aminotransferase

In order to survey the tissue distribution of 4 -aminobutyrate aminotransferase mRNA in flounder, distribution of 4-aminobutyrate aminotransferase in the flounder tissues was analyzed by the highly sensitive reverse transcription-polymerase chain reaction (RT-PCR) technique. Total RNA was isolated from the flounder tissues of the brain, liver, kidney and

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FLOUNDER  ----------------------------------------TMGCLATTHSKAIHKIDIPS  20
HUMAN     SKERGQRGFSQEELETCMINQAPGCPDYSILSFMGAFHGRTMGCLATTHSKAIHKIDIPS  205
PIG       SKERGQSAFSKEELETCMINQAPGCPDYSILSFMGAFHGRTMGCLATTHSKAIHKIDIPS  240
RAT       SKERGQRGFSKEELETCMVNQSPGCPDYSILSFMGAFHGRTMGCLATTHSKAIHKIDIPS  240
                                           ********************

FLOUNDER  FDWPIAPFPRLKYPLEEFVKENQQEEARCLEEVEDLIVKYRKKKKTVAGIIVEPIQSEGG  80
HUMAN     FDWPIAPFPRLKYPLEEFVKENQQEEARCLEEVEDLIVKYRKKKKTVAGIIVEPIQSEGG  265
PIG       FDWPIAPFPRLKYPLEEFVKENQQEEARCLEEVEDLIVKYRKKKKTVAGIIVEPIQSEGG  300
RAT       FDWPIAPFPRLKYPLEEFVTDNQQEEARCLEEVEDLNVKYRKKKRTVAGIIVEPIQSEGG  300
          ******************..*************** *******.***************

FLOUNDER  DNHASDDFFRKLRDISRKHGCAFLVDEVQTGGGSTGKFWAHEHWGLDDPADVMTFSKKMM  140
HUMAN     DNHASDDFFRKLRDIARKHGCAFLVDEVQTGGGCTGKFWAHEHWGLDDPADVMTFSKKMM  325
PIG       DNHASDDFFRKLRDISRKHGCAFLVDEVQTGGGSTGKFWAHEHWGLDDPADVMTFSKKMM  360
RAT       DNHASDDFFRKLRDIARKHGCAFLVDEVQTGGGCTGKFWAHEHWGLDDPADVMSFSKKMM  360
          ***************.****************.******************.******

FLOUNDER  TGGFFHKEEFRPNAPYRIFNTWLGDPPKNLLLAEVINIIKREDLLSNAAHAGKVLLTGPL  200
HUMAN     TGGFFHKEEFRPNAPYRIFNTWLGDPSKNLLLAEVINIIKREDLLNNAAHAGKALLTGLL  385
PIG       TGGFFHKEEFRPNAPYRIFNTWLGDPSKNLLLAEVINIIKREDLLSNAAHAGKVLLTGLL  420
RAT       TGGFFHKEEFRPSAPYRIFNTWLGDPSKNLLLAEVINIIKREDLLNNVAHAGKTLLTGLL  420
          ************.*************.******************.*.******.**** *

FLOUNDER  DLQARYPQFISRVRGRGTSCSFDTPDESIRNKL---------------------------  233
HUMAN     DLQARYPQFISRVRGRGTFCSFDTPDDSIRNKLILIARNKGVVLGGCGDKSIRFRPTLVF  445
PIG       DLQARYPQFISRVRGRGTFCSFDTPDESIRNKLISIARNKGVMLGGCGDKSIRFRPTLVF  480
RAT       DLQARYPQFVSRVRGRGTFCSFDTPDEAIRNKLILIARNKGVVLGGCGDKSIRFRPTLVF  480
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Fig. 3. Comparison of the deduced amino acid sequences of 4-aminobutyrate aminotransferase from flounder, human, pig, and rat.
The sequence of the flounder brain aminotransferase is aligned by using Clustal Multiple Alignment Program (Higgins and Sharp, 1989) with human, pig, and rat. *Asterisks* indicate a perfect match between all four sequences. *Dots* indicate a conversative replacement. The box (highlighted) designates a putative PLP binding site.

pancreas. Total RNA from various tissues was used as template to generate cDNA for RT-PCR amplification. RT-PCR was performed as described under Materials and Methods using the oligonucleotide primers (Table 1) and the isolated total RNA from various tissues, and analyzed on 1.5% agarose gel. As shown in Figure 4A, the expected DNA fragment (370 bp) was amplified from all tissue samples tested and the DNA banding patterns resulting from RT -PCR provided evidence for the expression of 4 -aminobutyrate aminotransferase. We detected the distribu-tion of mRNA in all the tissues tested in the brain, liver, kidney, and pancreas. This result strongly suggested that the tissues tested for mRNA have the transcription of the flounder 4-aminobutyrate aminotransferase.

In addition to the evidence from RT-PCR, patterns of the expression of flounder 4-aminobutyrate aminotransferase were also detected by Southern hybridization analysis (Figure 4B). The PCR products were transferred to a nitrocellulose membrane and hybridized with a random-primed DNA probe from a partial cDNA fragment of flounder 4-aminobutyrate aminotransferase. From the result of the Southern blot analysis, it is clearly evidented that the PCR generated fragments are derived from the flounder 4-aminobutyrate aminotransferase. From the RT-PCR data on the distribution of 4-aminobutyrate aminotransferase mRNA are consistent with previously reported data on the tissue and cellular distribution of 4-aminobutyrate aminotransferase. In addition to that, the derived amino acid sequence from a flounder partial cDNA clone contains a cofactor binding site, a pyridoxal-5-phosphate, which is very well conserved

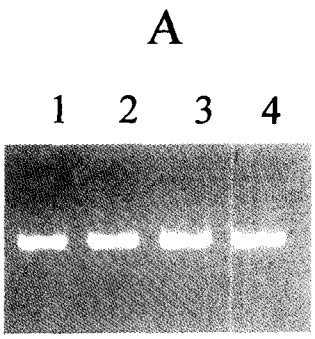

Fig. 4. Detection of flounder 4-aminobutyrate aminotransferase expression by RT-PCR (A) and Southern blot analysis (B). Poly(A^+) RNA from various tissues was used to generate cDNA for RT-PCR amplification. The PCR products were transferred to a nitrocellulose membrane and hybridized with a random-primed DNA probe from a partial cDNA fragment of flounder 4-aminobutyrate aminotransferase. Various tissues are indicated at lane 1 (brain), lane 2 (liver), lane 3 (kidney), and lane 4 (pancreas).

domain of aminotransferase and covalently bound to a lysyl residue of the enzyme. The amino acid sequence of the cofactor binding site was the same as that of previously reported mammalian 4-aminobutyrate aminotransferase, suggesting that microenvironmental structure of the cofactor binding region of 4-aminobutyrate aminotransferase, including fish, has been highly conserved during the evolution.

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