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The nucleotide and deduced amino acid sequences of porcine liver proline-β-naphthylamidase

Evidence for the identity with carboxylesterase

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A cDNA clone for porcine liver proline-β-naphthylamidase was isolated and sequenced. The deduced amino acid sequence of 567 residues was highly homologous with those of carboxylesterases (EC 3.1.1.1) previously reported for other species. In addition, proline-β-naphthylamidase purified from porcine liver was shown to have strong activity towards *p*-nitrophenylacetate, a representative substrate for carboxylesterases. These results suggest that proline-β-naphthylamidase is identical with carboxylesterase.

Proline-β-naphthylamidase; Carboxylesterase; cDNA cloning

1. INTRODUCTION

Proline-β-naphthylamidase has recently been purified from porcine intestinal mucosa and characterized [1–3]. It is composed of 3 identical subunits with a molecular weight of 58 000 each, and is a serine enzyme hydrolyzing some amino acid-β-naphthylamides, among which L-proline-β-naphthylamide is the best substrate [1,3]. It also hydrolyzes some ester substrates at greater rates than L-proline-β-naphthylamide [1]. On the other hand, carboxylesterase (EC 3.1.1.1) can hydrolyze various ester substrates such as *p*-nitrophenylacetate and was purified from microsomes of various organs such as liver, kidney and brain [4]. Among them, the porcine liver enzyme is the most extensively studied. It is composed of 3 subunits with a molecular weight of about 60 000 each and is a serine enzyme hydrolyzing various ester substrates and also some amide substrates at lower rates. Thus, carboxylesterase is very similar to proline-β-naphthylamidase in various characteristics. Further, we have become aware that the NH_2-terminal sequences of liver carboxylesterases from other species [5–7] are highly homologous with that of porcine intestinal proline-β-naphthylamidase [1].

In the present study, we intended to elucidate whether the two enzymes are identical or not. We, therefore, purified proline-β-naphthylamidase from the porcine liver microsome fraction and determined its NH_2-terminal amino acid sequence. Further, using its antisera, we isolated a cDNA clone encoding proline-β-naphthylamidase from a porcine liver λgt11 library and sequenced it. The deduced amino acid sequence was highly homologous with those of rat and rabbit liver carboxylesterases [5–7]. In addition, the activity towards *p*-nitrophenylacetate of the purified proline-β-naphthylamidase was confirmed. These results provide strong evidence for the identity of proline-β-naphthylamidase with carboxylesterase.

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin; kb, kilo base.

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2. MATERIALS AND METHODS

2.1. *Determination of enzyme activities and protein concentration*

Enzyme activities toward proline-β-naphthylamide (Sigma) and *p*-nitrophenylacetate (Sigma) were assayed essentially according to Takahashi et al. [1] and Heymann et al. [8], respectively. Protein concentration of enzyme solutions was determined by using a Bio-Rad protein assay kit.

2.2. *Polyacrylamide gel electrophoresis*

Slab gel electrophoresis in the presence or absence of SDS was performed by the method of Laemmli [9].

2.3. *Purification and NH_2-terminal sequence analysis of porcine liver proline-β-naphthylamidase*

All purification procedures were performed at 4°C. Microsomal fraction obtained from porcine liver homogenate was solubilized with 1% Triton X-100, and the resulting supernatant was used as crude enzyme extract. Further purification was performed essentially according to Takahashi et al. [1]. Amino acid sequence analysis of the

purified liver enzyme was performed by an automated protein sequencer (model 477A, Applied Biosystems) onlined to a PTH-amino acid analyzer (model 120A, Applied Biosystems).

2.4. *Preparation of antisera*

Two rabbits were immunized by subcutaneous injection with 0.5 mg each of the purified enzyme emulsified with an equal volume of Freund's complete adjuvant (Difco) 3 times at 2-week intervals. The antisera were obtained from the blood collected one week after the last injection.

2.5. *Screening of a cDNA library*

A porcine liver cDNA library in bacteriophage λgt11 (Clontech Laboratories) was screened essentially according to the method of Sambrook et al. [10] with *Escherichia coli* Y1090 as the host. In immunological screening, Hybond C (Amersham) was used as membrane for transferring phage plaques, vectastain ABC kit with anti-rabbit IgG secondary antibody (Vector) for detection and the antiserum against porcine liver proline-β-naphthylamidase as a probe. In screening by DNA hybridization, Hybond N (Amersham) was used as membrane for transferring phage plaques and an appropriate DNA fragment labelled with [α-^{32}P]dCTP (3000 Ci/mmol, ICN) by multiprime labelling kit (Amersham) as a probe. Fixation of DNA to the membrane, prehybridization, hybridization and washing were performed essentially as recommended by the manufacturer. The positive plaques obtained in both series of screenings were purified through one or two rounds of screening until the single positive plaque was picked up.

2.6. *Subcloning of λgt11 cDNA inserts and DNA sequencing*

Subcloning was performed essentially according to Sambrook et al. [10]. Recombinant bacteriophage DNA of each of the positive clones was purified from a lysate produced in a liquid LB medium. Appropriate inserts were subcloned in M13mp18 and transfections were performed with *E. coli* JM 103 as the host bacterium. Vector and insert DNAs were purified after alkaline lysis of the bacteria.

The purified DNAs were analyzed by restriction mapping and appropriate digestive fragments were further subcloned in M13mp18 or M13mp19. The single strand DNAs were purified and sequenced by the dideoxy termination method [11] using *Taq* DNA polymerase (Perkin-Elmer-Cetus) and a DNA sequencer (model 370A, Applied Biosystems).

3. RESULTS AND DISCUSSION

3.1. *Purification and the NH_2-terminal sequence of porcine liver proline-β-naphthylamidase*

Porcine liver proline-β-naphthylamidase was purified to homogeneity as examined by SDS-PAGE (data not shown). The characteristics of the purified enzyme such as specific activity, SDS-PAGE pattern and chromatographic behaviour on Sephacryl S-200 and Mono Q (Pharmacia) (data not shown) were essentially the same as those of the porcine intestinal enzyme [1].

The major sequence of the NH_2-terminal 22 residues of the porcine liver enzyme was Gly-Gln-Pro-Ala-Ser-Pro-Pro-Val-Val-Lys/Asp-Thr-Ala-Gln-Gly-Arg/Leu-Val-Leu-Gly-Lys-Tyr-Val-Ser. At positions 10 and 15, two major PTH-amino acids were detected, probably due to isoforms of the enzyme. Compared to the porcine intestinal enzyme [1], three residue substitutions were observed: Ser^5 ↔ Val, Pro^7 ↔ Ser, and Lys^{19} ↔ Arg. These differences are thought to be due to organ-specific isoforms.

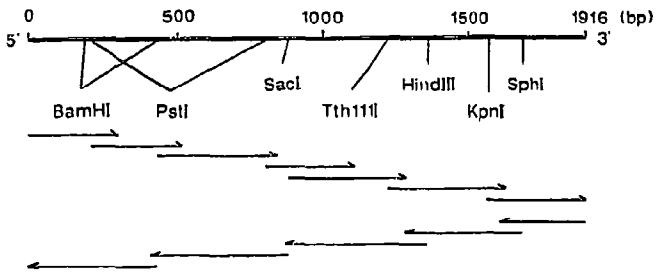

Fig. 1. Sequencing strategy for a cDNA clone, PLCE-A15, of porcine liver proline-β-naphthylamidase. The restriction sites used for subcloning of PLCE-A15 in M13mp18 or M13mp19 are shown. Arrows indicate directions and extents of nucleotide sequencing of restriction fragments.

3.2. *Isolation and sequence determination of porcine liver proline-β-naphthylamidase cDNA*

By immunological screening, 5 positive clones were isolated from 5×10^4 clones. Among them, a clone named PLCE-6 had the longest insert cDNA of 1.2 kb. By rescreening with this cDNA as a probe, 36 clones with rather strong positive signals were obtained from 9×10^4 clones. Among them, a clone named PLCE-25 had the longest insert of 1.7 kb. This clone was found to lack the 5'-end of the coding region of the intact cDNA. Therefore, the 1.2 kb of *Hin*dIII fragment containing the 5'-end of the insert cDNA of PLCE-25 was used for further screening. After screening of 5×10^5 clones, a clone with 1.9-kb insert (PLCE-A15) was isolated and sequenced. The sequence strategy and the nucleotide and deduced amino acid sequences are shown in Figs. 1 and 2, respectively. The cDNA had 1916 bp, including a 1698-bp open reading frame starting with ATG (positions 6–8) and ending with TGA (positions 1704–1706) with a poly A tail at the 3'-end. The deduced amino acid sequence from positions 19 to 40 agreed with the NH_2-terminal sequence of the mature protein determined at the protein level, and the calculated molecular weight (60 056) of the mature protein also agreed well with the molecular weight (58 000) of each subunit of the purified protein. The cDNA clone was, therefore, confirmed to encode porcine liver proline-β-naphthylamidase. Besides, the mature protein was presumed to be produced from the pre-protein by liberating a signal peptide of the NH_2-terminal 18 amino acid residues.

3.3. *Comparison of the amino acid sequence of porcine liver proline-β-naphthylamidase with those of liver carboxylesterases from other species*

The deduced amino acid sequence of porcine liver proline-β-naphthylamidase was highly homologous with the amino acid sequences of rat [5] and rabbit [6,7] liver carboxylesterases; the identity being 76.2% with rat liver carboxylesterase and 73.0% and 46.9% with rabbit liver carboxylesterase isoforms 1 and 2, respectively (Fig. 3). Ser^{204} and His^{449} are common to these enzymes

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CCAAGATGTGGCTTCTCCCGCTGGTCCTGACCTCCCTCGCCTCTTCTGCAACTTGGGCAGGGCAGCCAGCCTCGCCGCCTGTTGTGGACACTGCC    95
    MetTrpLeuLeuProLeuValLeuThrSerLeuAlaSerSerAlaThrTrpAlaGlyGlnProAlaSerProProValValAspThrAla      30

CAGGGCCGAGTCCTGGGGAAGTACGTCAGCTTAGAAGGCCTGGCACAGCCGGTGGCCGTCTTCCTGGGAGTCCCTTTTGCCAAGCCCCCTCTCGGA   191
GlnGlyArgValLeuGlyLysTyrValSerLeuGluGlyLeuAlaGlnProValAlaValPheLeuGlyValProPheAlaLysProProLeuGly    62

TCCTTGAGGTTTGCTCCGCCGCAGCCTGCAGAACCATGGAGCTTCGTGAAGAACACCACCTCCTACCCTCCCATGTGCTGCCAGGACCCAGTAGTG   287
SerLeuArgPheAlaProProGlnProAlaGluProTrpSerPheValLysAsnThrThrSerTyrProProMetCysCysGlnAspProValVal    94

GAGCAGATGACCTCAGATCTATTTACCAACGGAAAGGAGAGGCTCACTCTGGAGTTTTCTGAAGACTGTCTCTACCTAAATATTTACACCCCTGCT   383
GluGlnMetThrSerAspLeuPheThrAsnGlyLysGluArgLeuThrLeuGluPheSerGluAspCysLeuTyrLeuAsnIleTyrThrProAla   126

GACCTGACAAAGAGGGGCAGACTGCCGGTGATGGTGTGGATCCACGGAGGAGGCCTGGTGTTGGGCGGGGCACCAATGTATGATGGGGTGGTGCTT   479
AspLeuThrLysArgGlyArgLeuProValMetValTrpIleHisGlyGlyGlyLeuValLeuGlyGlyAlaProMetTyrAspGlyValValLeu   158

GCTGCGCATGAAAACGTGGTGGTGGTGGCCATCCAGTACCGCCTGGGCATCTGGGGATTCTTCAGCACAGGGGATGAACACAGCCGGGGCAACTGG   575
AlaAlaHisGluAsnValValValValAlaIleGlnTyrArgLeuGlyIleTrpGlyPhePheSerThrGlyAspGluHisSerArgGlyAsnTrp   190

GGTCACTTGGACCAGGTGGCCGCACTGCACTGGGTCCAGGAGAACATCGCCAACTTTGGAGGCGACCCAGGCTCTGTGACCATCTTTGGAGAGTCA   671
GlyHisLeuAspGlnValAlaAlaLeuHisTrpValGlnGluAsnIleAlaAsnPheGlyGlyAspProGlySerValThrIlePheGlyGluSer   222

GCAGGAGGGGAAAGTGTCTCTGTTCTGGTGTTGTCTCCCTTGGCCAAGAACCTCTTCCACCGGGCCATCTCTGAGAGTGGCGTGGCCCTCACTGTT   767
AlaGlyGlyGluSerValSerValLeuValLeuSerProLeuAlaLysAsnLeuPheHisArgAlaIleSerGluSerGlyValAlaLeuThrVal   254

GCCCTGGTCAGGAAGGACATGAAGGCTGCAGCTAAGCAAATTGCTGTCCTTGCTGGGTGTAAAACCACCACCTCGGCTGTCTTTGTTCACTGCCTG   863
AlaLeuValArgLysAspMetLysAlaAlaAlaLysGlnIleAlaValLeuAlaGlyCysLysThrThrSerAlaValPheValHisCysLeu   286

CGCCAGAAGTCGGAGGACGAGCTCTTGGACTTAACGCTGAAGATGAAATTTTTAACTCTTGATTTTCATGGAGACCAAAGAGAGAGCCATCCCTTC   959
ArgGlnLysSerGluAspGluLeuLeuAspLeuThrLeuLysMetLysPheLeuThrLeuAspPheHisGlyAspGlnArgGluSerHisProPhe   318

CTGCCCACTGTGGTGGATGGAGTGCTGCTGCCCAAGATGCCTGAAGAGATTCTGGCTGAGAAGGATTTCAACACTGTCCCCTACATCGTGGGAATC  1055
LeuProThrValValAspGlyValLeuLeuProLysMetProGluGluIleLeuAlaGluLysAspPheAsnThrValProTyrIleValGlyIle   350

AACAAGCAAGAGTTTGGCTGGCTTCTGCCAACGATGATGGGCTTCCCCCTCTCTGAAGGCAAGCTGGACCAGAAGACGGCCACGTCACTCCTGTGG  1151
AsnLysGlnGluPheGlyTrpLeuLeuProThrMetMetGlyPheProLeuSerGluGlyLysLeuAspGlnLysThrAlaThrSerLeuLeuTrp   382

AAGTCCTACCCCATCGCTAACATCCCTGAGGAACTGACTCCAGTGGCCACTGACAAGTATTTGGGGGGGACAGACGACCCCGTCAAAAAGAAAGAC  1247
LysSerTyrProIleAlaAsnIleProGluGluLeuThrProValAlaThrAspLysTyrLeuGlyGlyThrAspAspProValLysLysLysAsp   414

CTGTTCCTGGACTTGATGGGGGATGTGGTGTTTGGTGTCCCATCTGTGACGGTGGCCCGTCAACACAGAGATGCAGGAGCCCCCACCTACATGTAT  1343
LeuPheLeuAspLeuMetGlyAspValValPheGlyValProSerValThrValAlaArgGlnHisArgAspAlaGlyAlaProThrTyrMetTyr   446

GAGTTTCAGTATCGCCCAAGCTTCTCATCGGACAAGAAACCCAAGACGGTGATCGGGGACCACGGGGATGAGATCTTCTCCGTCTTTGGTTTTCCA  1439
GluPheGlnTyrArgProSerPheSerSerAspLysLysProLysThrValIleGlyAspHisGlyAspGluIlePheSerValPheGlyPhePro   478

CTGTTAAAAGGCGATGCCCCAGAAGAGGAGGTCAGTCTCAGCAAGACGGTGATGAAATTCTGGGCCAACTTTGCTCGCAGTGGGAACCCCAATGGG  1535
LeuLeuLysGlyAspAlaProGluGluGluValSerLeuSerLysThrValMetLysPheTrpAlaAsnPheAlaArgSerGlyAsnProAsnGly   510

GAGGGGCTGCCCCATTGGCCGATGTACGACCAGGAAGAAGGGTACCTTCAGATCGGCGTCAACACCCAGGCAGCCAAGAGGCTGAAAGGTGAAGAA  1631
GluGlyLeuProHisTrpProMetTyrAspGlnGluGluGlyTyrLeuGlnIleGlyValAsnThrGlnAlaAlaLysArgLeuLysGlyGluGlu   542

GTGGCCTTCTGGAACGATCTCCTGTCCAAGGAGGCAGCAAAGAAGCCACCCAAGATAAAGCATGCTGAGCTGTGAAGGGGAGGCTCGGCTGACCTT  1727
ValAlaPheTrpAsnAspLeuLeuSerLysGluAlaAlaLysLysProProLysIleLysHisAlaGluLeu***

GAGGGCTTGTGGGACCGCAGGCAGGAGCAGTCTACCGAAGGCGTTCATGGGCCAGGAGGTATCTTCTTGTGGAGGCTGGGGAATTAGCGGGTGGGA  1823

GTGGCGGAGATCAGGGAGGGGGAATTTCTGTAGCTGCGGCCTCAATTTTAGAAATAAATGTTCTTTTGGAGACCAAAAAAAAAAAAAAAAAAAA
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Fig. 2. The nucleotide and deduced amino acid sequences of PLCE-A15. The underlined amino acid sequence was identical with the NH_2-terminal amino acid sequence of the purified enzyme. The in-frame stop codon (TGA) is labelled '***'.

and are thought to be the active site residues as presumed in the case of rabbit liver carboxylesterase isoform 1 [6]. The sequences around these residues are also well conserved. The COOH-terminal tetrapeptide, H X E L, considered to be related to protein localization to the luminal side of endoplasmic reticular membranes [5-7], are also conserved in these 4 enzymes. These results

Fig. 3. Comparison of the deduced amino acid sequence of porcine liver proline-β-naphthylamidase with those of rat and rabbit liver carboxylesterases. The amino acids are shown in single letter code. Amino acid residues corresponding to porcine liver proline-β-naphthylamidase are numbered in the right-hand margin. Hyphens indicate gaps introduced for maximal homology. Amino acids common with the proline-β-naphthylamidase are labelled '·'; histidine and serine residues at the putative active site are labelled'+'; COOH-terminal tetrapeptides are shown in **bold** face. 'porc.' and 'rat' mean the predicted amino acid sequences of porcine liver proline-β-naphthylamidase and rat liver pI 6.1 esterase [5], respectively. 'rab.1' and 'rab.2' are rabbit liver carboxylesterase form 1 [6] and form 2 [7], respectively.

strongly suggest the identity of proline-β-naphthylamidase with carboxylesterase.

3.4. *Activities toward proline-β-naphthylamide and p-nitrophenylacetate of the purified porcine liver proline-β-naphthylamidase*

When the purified porcine liver proline-β-naphthylamidase was electrophoresed in 4.5% polyacrylamide gel without SDS and stained with Coomassie brilliant blue, a rather broad protein band was obtained (Fig. 4), which is consistent with the presence of multiple (at least 4 and possibly more than 10) isoforms [4,12]. After electrophoresis, the gel was sliced into 2-mm thick sections and activities toward proline-β-naphthylamide and *p*-nitrophenylacetate (a representative substrate for carboxylesterase) in the extract of each slice were assayed. The protein band stained with Coomassie brilliant blue and the 2 activities coincided almost completely (Fig. 4), suggesting that the 2 activities are inherent to a single enzyme with multiple isoforms.

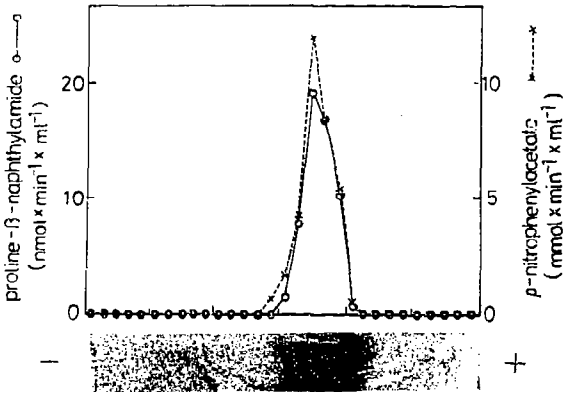

Fig. 4. Activities toward proline-β-naphthylamide and p-nitrophenylacetate in gel slices after polyacrylamide gel electrophoresis of the purified enzyme. Approximately 5 μg of proline-β-naphthylamidase purified from porcine liver was electrophoresed in 4.5% polyacrylamide gel without SDS. The activities toward the two substrates in the gel slices are shown as positions corresponding to the gel stained with Coomassie brilliant blue.

Thus, the results obtained in the present study provide strong evidence for the identity of proline-β-naphthylamidase and carboxylesterase.

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