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Identification and Characterization of a Mouse Dipeptidase That Hydrolyzes L-Carnosine

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L-Carnosine is a bioactive dipeptide present in mammalian tissues including the central nervous system. We have recently shown that L-carnosine is involved in the regulation of energy homeostasis through the autonomic nervous system, but the mechanisms for its biosynthesis and degradation have not yet been fully elucidated. Here we report the biochemical and immunohistochemical characterization of a mammalian protein that has a 17% overall amino acid sequence homology with a *Lactobacilus* carnosinase, PepV. A recombinant protein expressed in E. coli has the enzymatic ability to digest L-carnosine and various other dipeptides, and this activity is inhibited by bestatin. It requires Mn^{2+} for enzymatic activity and its effect is reversible. Immunohistochemical analysis showed that a few neuronal populations express this protein at very high levels. It is highly expressed in the parafascicular nucleus of the thalamus, tuberomammillary nucleus of the hypothalamus and the mitral cell layer of the olfactory bulb. In addition, neuronal processes, but not cell bodies, are stained in the striatum. In all these areas, the protein did not colocalize with the glial fibrilary acidic protein. These results suggest that a peptidase that digests L-carnosine is enriched in several specific neuronal populations in the central nervous system.

Key words: carnosinase, L-carnosine, dipeptidase, metaloproteinase, M20 family, CN2.

Mammalian tissues have several dipeptides containing L-histidine or its derivatives, such as L-carnosine (β-alanyl-L-histidine), homocarnosine (γ-aminobutyl-L-histidine) and anserin (β-alanyl-*N*-methyl-L-histidine) (*1*). L-carnosine is present at high concentrations in the skeletal muscles (>10 mM) and the nervous system (>1 mM). In the nervous system, it is present in the olfactory epithelium and neurons of the olfactory bulb, as well as in glial cells of other brain areas (*2*). L-Carnosine–like immunoreactivity has also been found in several neurons of the hypothalamic suprachiasmatic nucleus and the anterior hypothalamus (*3*), suggesting that L-carnosine might be involved in the olfactory system and the hypothalamic neuronal networks.

We have recently found that L-carnosine suppresses hyperglycemia induced by an intracranial injection of 2-deoxy-D-glucose (2DG-hyperglycemia) (*4*). This effect of L-carnosine is blocked with thioperamide, an antagonist of the histamine H3 receptor, suggesting that the pathway of L-carnosine action might have a direct or indirect connection with the histamine H3 receptor. This effect seems to be mediated by the autonomic nervous system because the central administration of L-carnosine suppresses sympathetic nerve activities and activates vagal activities (*5*). In addition, L-carnosine suppresses sympathetic nerve activity in DOCA-induced hypertensive rats (*6*). These findings indicate that L-carnosine is involved in the central regulation of blood glucose and blood pressure homeostasis through the autonomic nervous system.

Although L-carnosine is a classical dipeptide, the molecular mechanisms of its synthesis and degradation are largely unknown. Carnosinases are the enzymes that hydrolyze L-carnosine to yield β-alanine and L-histidine. We have recently found that carnosinase activity in the plasma, as well as L-carnosine synthetase activity in the skeletal muscles, undergoes daily changes in the rat (*6*). In addition, plasma carnosinase activity is down regulated by exercise on a running wheel (*6*). These findings indicate that L-carnosine synthesis and degradation are regulated under various physiological conditions.

In mammals, at least two types of carnosinases with different properties are separated by column chromatography (*7*, *8*). One hydrolyzes a variety of dipeptides including L-carnosine, but does not hydrolyze homocarnosine or anserin. This enzyme requires Mn^{2+} ions for its activity and is strongly inhibited by low concentrations of bestatin (*8*). The other carnosinase hydrolyzes not only L-carnosine but also homocarnosine and anserine, and these activities are not inhibited by bestatin (*8*).

The peptidase M20 family is a subfamily of metalloproteinases containing peptidic bond-hydrolyzing enzymes that exist in a wide variety of species from bacteria to mammals (*9*). Among them, pepV from Lactobaccilus has been shown to have L-carnosine hydrolyzing activity (*10*). To find a mammalian enzyme that hydrolyzes L-carnosine, we searched a mammalian cDNA database for cDNAs showing sequence homology with Lactobacilus pepV, and found a cDNA encoding a protein sharing 17% amino acid sequence homology with PepV that encodes a possible cytosolic protein of 52 kDa without a signal sequence. We have confirmed that the protein product has carnosinase activity using the purified recombinant

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protein, and have designated it as MCP1 (Mouse carnosinase homologous to PepV). During the preparation of a paper, two dipeptidases digesting L-carnosine were reported and designated as CN1 and CN2 (*11*), and the primary sequence of CN2 matches that of MCP1. It was reported that CN2 has dipeptide-degrading activity with a wide spectrum of substrate specificity, digesting a variety of dipeptides including carnosine, but that it does not digest homocarnosine. Its activity was reported to be sensitive to bestatin, an inhibitor of peptidases, including several types of carnosinases. These findings suggest that CN2 is a cytosolic dipeptidase in mammalian tissues.

In the present work, we examined the biochemical characteristics of this protein and, to obtain further insight into its physiological functions, especially in the mammalian nervous system, examined its immunohistochemical distribution in rat brain. In this paper, we call this protein CN2.

EXPERIMENTAL PROCEDURES

Antibodies—Rabbit anti-CN2 antiserum was raised against recombinant full length carnosinase prepared as described below. Horseradish peroxidase–labeled goat anti-rabbit antibody was purchased from Cell Signaling.

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)—Total RNA was prepared from mouse brains using guanidinium thiocyanate (*12*), and the mRNA was purified from the total RNA using oligo dT beads (Oligotex dT30, Takara). The cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen) using oligo dT (*18*) as a primer and the purified mRNA as a template. Then the open reading frame of RIKEN cDNA, NM_023149, encoding a putative mouse carnosinase (MCP1 or CN2) was amplified by polymerase chain reaction with synthetic DNA primers, 5′-CGAATTCGATGTCAGCCCTCAAAGCTGT-3′ and 5′-GCACGTGTCAGTTCTTCAGCT-3′, complimentary to the 3′ and 5′ ends of the open reading frame, respectively. An amplified fragment of 1.2 kbp was subcloned into pBluescript II vector and its DNA sequence determined.

Expression of Recombinant CN2—The cDNA encoding CN2 was subcloned into pGEX-4T3 vector (Amersham). The CN2 protein fused with glutathione-S-transferase was induced in E.Coli, strain BL21(DE3), in the presence of 1 mM IPTG for 12–16 h at 25°C. The bacteria were then collected and sonicated in buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1mM EDTA and 5 mM 2-mercaptoethanol. After centrifugation, the supernatant was mixed with glutathione-Sepharose beads (Amersham), washed, and incubated with thrombin (Amersham) at 25°C for 12–16 h. The soluble fraction was applied to an anion exchange column (Hi-trap Q, Amersham), and the proteins were eluted with a linear gradient of 50–300 mM NaCl in the same buffer.

Measurement of Carnosinase Activity by HPLC—The recombinant protein (0.2–1 μg) was mixed with L-carnosine in buffer containing 10 mM Tris-HCl, pH 8.8, and 2.5 mM DTT, 10 μM $MnCl_2$ and incubated for 30 min at 37°C unless otherwise indicated. The reaction was stopped by adding an equal volume of 10% trichloroacetic acid and centrifuging. The supernatant was then fractionated by HPLC using a cation-exchange column (Capcellpak SCX, 4.6 × 150 mm, Shiseido). L-carnosine and histidine concentrations were estimated by absorbance at 215 nm.

Thin Layer Chromatography—Samples were spotted onto a cellulose plastic sheet for thin layer chromatography (Merck), and separated with formic acid/isopropyl alcohol/water (10:30:60, v/v). The plate was then air dried, and amines were visualized by spraying with ninhydrin solution.

Preparation of Tissue Extracts—Tissues were excised from female Wistar rats weighing 200–300 g and homogenized in 10 volumes of a buffer containing 1% NP40, 150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4). After centrifugation at 15,000 rpm for 20 min, the supernatants were recovered and protein concentrations were determined by the method of Bradford.

Western Blotting—Tissue extracts containing 10 mg of proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to a nitrocellulose membrane. After blocking with 1% Tween 20 in 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA (Tween-TBS), the membrane was incubated with a primary antibody diluted 1:1,000 with Tween-TBS, washed, and incubated with horseradish peroxidase–conjugated anti-rabbit IgG. Immunoreactive bands were visualized with a chemiluminescence reagent (PerkinElmer).

Immunohistochemical Staining—Rats were anesthetized with sodium pentobarbital, perfused with phosphate-buffered saline (PBS), and then with PBS containing 4% paraformaldehyde. The brains were removed, post-fixed at 4°C for 2 days, cryoprotected with 30% sucrose at 4°C for 5 days, and cut into 20-μm sections with a cryostat. The sections were treated for 3 h in PBS containing 3% bovine serum albumin and 0.3% Triton X-100, and incubated overnight with primary antibodies to anti-carnosinase diluted in Tween/TBS. Immunoreactive signals were visualized by the avidin-biotin system (Vector, UK) with 3,3′-diaminobenzidine (Dojindo Laboratories, Kumamoto, Japan) as the substrate, and observed using a conventional microscope system. Images were taken with a CCD camera attatched to the microscope. For double-staining analysis, fluorescein-labeled anti-mouse IgG and rhodamine-labeled anti-rabbit IgG were used as secondary antibodies, and the fluorescence signals were observed using a Zeiss confocal laser scanning microscope system.

RESULTS

In order to identify a DNA encoding a rodent carnosinase, we searched for a cDNA showing sequence homology with Lactobacillus carnosinase, pepV (*10*). A psyBLAST search (*13*) against the NCBInr DNA database showed that a mouse cDNA, NM_023149, which was originally isolated by the RIKEN cDNA project (*14*) (mus musculus RIKEN cDNA 060020E05A), has 17% overall sequence identity with pepV (Fig. 1A). This cDNA has an open reading frame encoding a putative protein product 476 amino acids in length.

Sequence motif analysis using PROSITE (*15*) showed that the protein contains a consensus sequence of the peptidase M20 family. This family is composed of a variety of enzymes that hydrolyze the peptidic bonds of small molecules such as disuccinyldiaminopimelate desuccinyl-

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(A)

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pepV          --MDLNFKELAEAKKDAILKDLEELIAIDSSEDLENATEEYPVGKGPVDAMTKFLS----
NM_023149     MSALKAVFQYIDENQDRYVKKLAEWVAIQSVSAWPEKRGEIRRMMEVAAADVQRLGGSVE
                    . :  : ::*  :*.* * :**:* .   :   *       . * .: *.

pepV          ------FAKRDGFDTENFANYAGRVNFGAGDKRLGIIGHMDVVPAG--EGWTRDPFKMEI
NM_023149     LVDIGKQKLPDGSEIPLPPILLGKLGSDPQKKTVCIYGHLDVQPAALEDGWDSEPFTLVE
                       ** :    .   *::. .. .* : * **:** **.  :**  :**.:

pepV          DEEGRIYGRGSADDKGPSLTAYYGMLLLKEAGFKPKKKIDFVLGTNEETNWVGIDYYLKH
NM_023149     RE-GKLYGRGSTDDKGPVAGWMNALEAYQKTGQEIPVNLRFCLEGMEESGSEGLDELIFA
               * *::*****:*****      .:   :::* :   :: * *   **:.  *:*  :

pepV          EPTPDIVFSPDAEYPIINGEQGIFTLEFSFKNDDTKGDYVLDKFKAGIATNVTPQVTRAT
NM_023149     QKD---KFFKDVDYVCISDNYWLGKNKPCITYGLRGICYFFIEVECSDKD-----LHSGV
              :      *  *.:*  *..:  : . : .:. .     *.: :.:..        :  ..

pepV          ISGPDLEAVKLAYESFLADKELDGSFEINDESADIVLIGQGAHASAPQVGKNSATFLALF
NM_023149     YGGSVHEAMTDLISLMGCLVDKKGKILIPGINDAVAPVTDEEHALYDHIDFDMEEFAKDV
               .*.  **:.   . : .  : .*.: * . .  :. : :  **   ::. :   *   .

pepV          LDQYAFAGRDKNFLHFLAEVEHEDFYGKKLGIFHHDDLMGDLASSPSMFDYEHAGKASLL
NM_023149     GAETLLHSCKKDILMHRWRYPSLSLHGIEG----AFSGSGAKTVIPRKVVGKFSIRLVPD
                :  : . .*::* .  .    .::* :       .  *  :  *  .  :.: :

pepV          NNVRYPQGTDPDTMIKQVLDKFSGILDVTYNGFEEPHYVPGS-DPMVQTLLKVYEKQTGK
NM_023149     MIPEVVSEQASSYLSKKFAELQSPNKFKVYMGHGGKPWVSDFNHPHYQAGRRALKTVFGV
                 .  .   .. : *:. :  *     .* *.    :*..  .*  *:  :. :.  *

pepV          PGHEVVIGG---GTYGRLFERGVAFGAQPE-NGPMVMHAANEFMMLDDLILSIAIYAEAI
NM_023149     EPDLTREGGSIPVTLTFQEATGKNVMLLPVGSADDGAHSQNEKLNRLNYIEGTKMLAAYL
                . .  **    *       *  .   *  ..    *: ** :   : * .  : *  :
pepV          YELTKDEEL
NM_023149     YEVSQLKN-
              **::: ::
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(B)

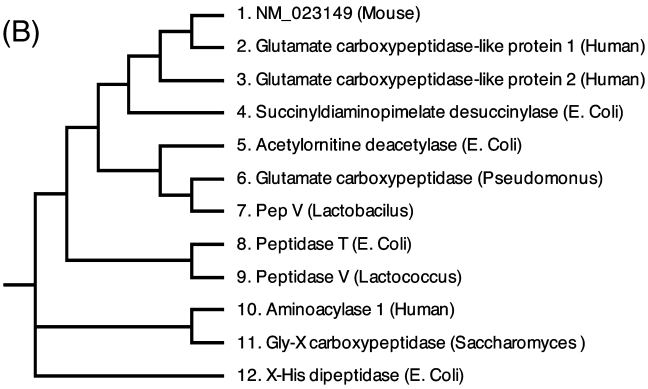

Fig. 1. **Primary structure of a putative carnosinase, NM_023149.** (A) A psy-Blast search showed that RIKEN cDNA 060020E05A (NCBI accession No. NM_023149) has sequence homology with *Lactobacillus* pepV. The deduced amino acid sequence of the open reading frame encoded by NM_023149 was aligned with pepV using ClustalW, showing 17% sequence identity and 47% similarity. (B) Phylogenic tree of NM_023149 and related proteins. Amino acid sequences of NM_023149 and other peptidase M20 family members were aligned and a phylogenic tree created using ClustalW. The results was converted to tree form using TreeviewX. The database accession number of each DNA is as follows. 1, NM_023149; 2, Swissprot Q96KP4; 3, Swissprot Q96KN2; 4, Swissprot P24176; 5, Swissprot Q8FB97; 6, Swissprot P06621; 7, NCBI CAA83252; 8, Swissprot P29745; 9,Swissprot L27596; 10, Swissprot Q03154; 11, Swissprot P27614; 12, Swissprot P15288.

lase (dapE) (*16*), aminoacylase (acy1) (*17*, *18*), acetylornitine deacetylase (argE) (*19*), carboxypeptidase G2 (*20*), and tripeptidase (pepT) (*21*). The phylogenetic tree of these enzymes is shown in Fig. 1B. Among these proteins, NM_023149 is most closely related to a putative peptidase, human glutamate carboxypeptidase–like protein 1, showing 99% amino acid sequence homology, indicating that it is a human counterpart of NM_023149. This subfamily also includes human glutamate carboxypeptidase–like protein 2, which has a signal peptide for secretion.

In order to confirm that this gene is actually expressed in mouse tissues, we prepared mRNAs from rat brain and performed a reverse transcriptase–polymerase chain reaction (RT-PCR) using a pair of primers corresponding to the 5′ and 3′ ends of the open reading frame of NM_023149. This resulted in a single band of 1.5 kbp, which is consistent with the length of the open reading frame, 1,385 bp.

In order to characterize the protein product, the cDNA amplified by RT-PCR was subcloned into pGEX 4T-3 vector and a corresponding GST-fusion protein was expressed in *E. coli* (Fig. 2A). After the addition of IPTG, a 70k protein was induced and recovered in the soluble fraction of E.Coli extracts. The fusion protein was then purified by glutathione-Sepharose column chromatography and digested with thrombin to yield the full length protein without a GST-tag. This protein was further purified by anion exchange chromatography. The purified product appeared as a 52 kDa protein with no other detectable bands on a Coomassie Brilliant Blue-stained gel. The apparent molecular weight was consistent with that calculated from the cDNA sequence, 52,739.

To confirm that this protein had carnosinase activity, the purified protein was incubated with L-carnosine and the resultant reaction products were separated by HPLC on a cation exchange column. When column eluates were monitored by absorbance at 215 nm, an absorbance peak whit a retention time corresponding to that of histidine increased dependent on substrate concentrations, indicating that L-carnosine was enzymatically digested with the release of histidine. In this system, β-alanine was undetectable because it does not have absorbance at 215 nm. However, thin layer chromatography analysis showed

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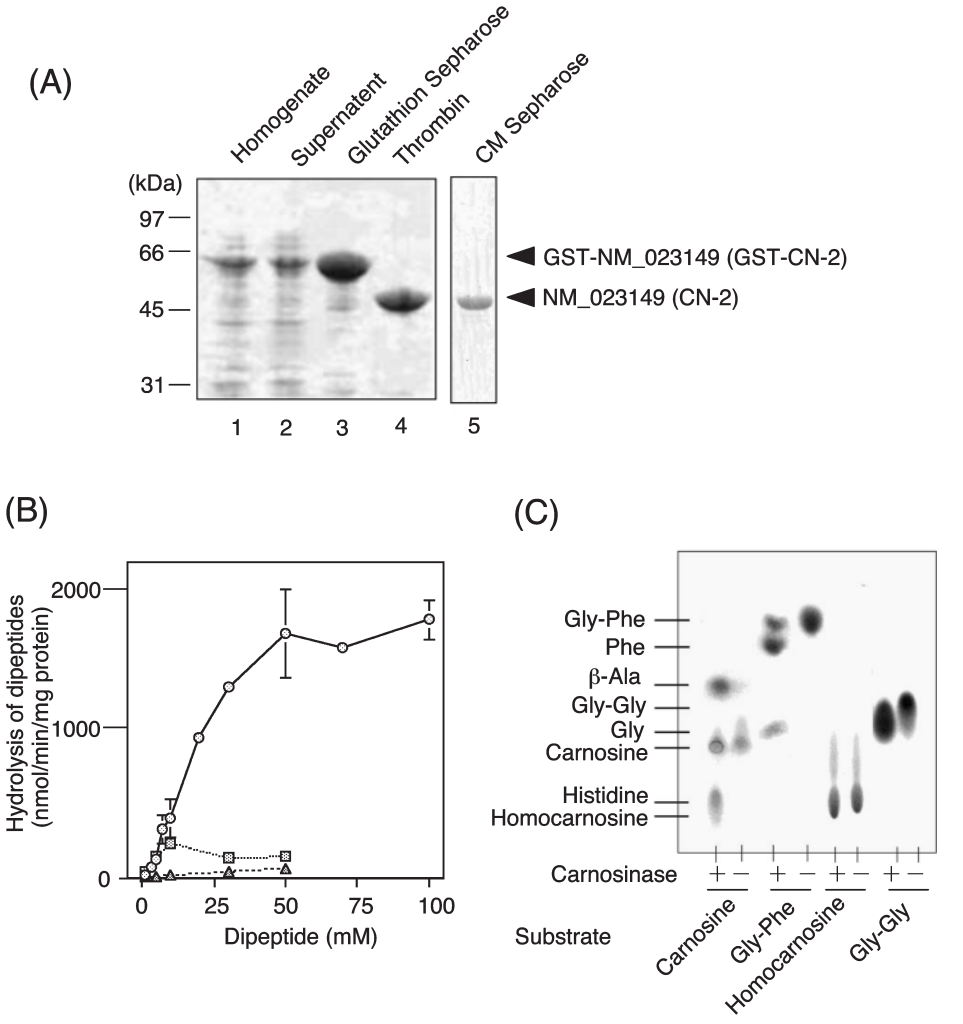

Fig. 2. **Expression of recombinant CN2 and its enzymatic activity.** (A) mRNA was purified from rat brain and the cDNA for NM_023149 (CN2) was amplified by RT-PCR. A 1.5 kbp DNA product was subcloned into pGEX 4T3 vector and the corresponding GST-fusion protein was expressed in *E. coli* (Lane 1). The supernatant fraction of the crude bacterial extract (Lane 2) was applied to a glutathione Sepharose column (Lane 3), and the full length protein without the GST-tag was eluted by digestion with thrombin (Lane 4). The eluted protein was further purified by ion exchange chromatography on a Q-Sepharose column (Lane 5). (B) The recombinant CN2 protein was incubated with the indicated concentrations of L-carnosine (open circles), anserine (open triangles) or homocarnosine (open squares) at 37 °C for 30 min. After TCA precipitation, the supernatants were separated by cation exchange HPLC. The eluted materials were monitored by absorbance at 215 nm (C) The recombinant CN2 protein was incubated with L-carnosine or other related molecules as indicated at 37 °C for 30 min. After TCA precipitation, samples were spotted onto a silica gel plate for thin layer chromatography and developed with a mixture of organic solvents as described in "EXPERIMENTAL PROCEDURES." Peptides and amino acids were detected by ninhydrin reaction.

that the major reaction products were histidine and β-alanine (Fig. 2C). These results confirm that the protein product of NM_023149 gene has carnosinase activity.

Using this assay system, we also examined whether this enzyme could digest homocarnosine (GABA-His). However, as shown in Fig. 2B, there was no peptidase activity for homocarnosine, even at high concentrations.

To examine substrate specificity, several peptides, including Gly-Gly, Gly-Phe, homocarnosine and L-carnosine, were tested by thin layer chromatography (Fig. 2C). Among them, Gly-Gly, Gly-Phe and L-carnosine were digested into the corresponding amino acids; however, consistent with the results described above, homocarnosine was not degraded. In addition, a tripeptide, Gly-Gly-Gly, was not degraded either (data not shown).

When we isolated this gene, it was an uncharacterized cDNA found only in a cDNA database, but it has since been independently isolated by Teufel et al. as an enzyme digesting carnosine as well as other dipeptides and designated CN2 (*11*). CN2 has been shown to have broad substrate specificity, and our results are consistent with this report. Therefore, we refer to this protein as CN2 hereafter.

It has been known that there are two types of carnosinase in mammalian tissues and serum (*8*). These two carnosinases have different sensitivities to the peptidase inhibitor bestatin. As shown in Fig. 3A, CN2 shows less than 20% of full activity in the presence of 1 μM bestatin. In addition, CN2 does not have a typical signal sequence essential for secretion. These findings are consistent with the report of Taufel *et al.* (*11*), and indicate that CN2 has several characteristics of the enzyme known as carnosinase.

In addition, we examined the effect of homocarnosine on the carnosinase activity of CN2 (Fig. 3A). Homocarnosine is structurally related to L-carnosine, but is not digested by CN2 as described above. When homocarnosine was present in the assay mixture, the activity of CN2 decreased to about 50% of full activity. This indicates that homocarnosine can interact with CN2 and weakly inhibit its activity.

Peptidase M20 comprises a family of metalloproteinases that require a divalent cation for activity. The standard assay mixture for the experiments described above contained Mn^{2+} as a metal source. In contrast, CN2 loses its activity in the presence of EDTA (Fig. 3B), confirming that CN2 requires a metal ion. To examine the metal ion selectivity, we incubated CN2 with EDTA to remove divalent cations, and, after dialysis, the activity was assayed in the presence of various metal ions. The results showed

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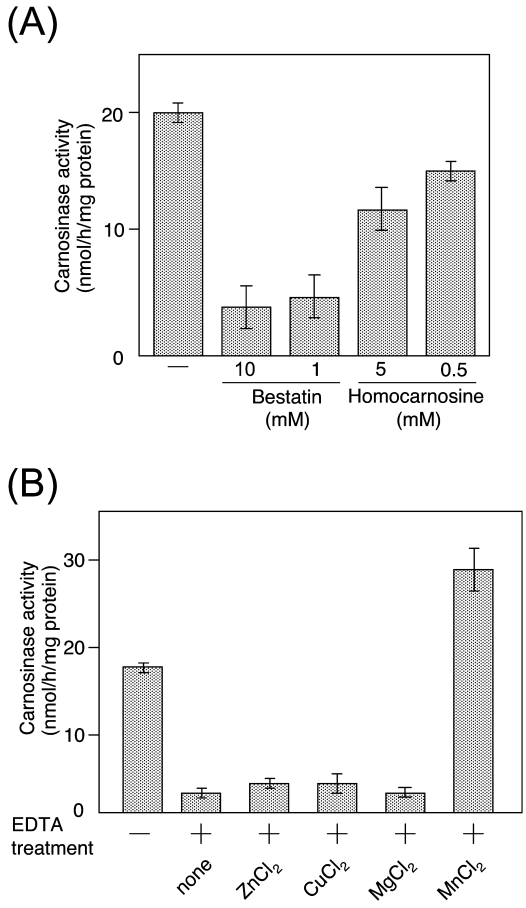

Fig. 3. **Effects of various reagents on the activity of CN2.** (A) The recombinant CN2 protein was incubated with L-carnosine together with bestatin or homocarnosine as indicated. Carnosinase activity was measured as described in the legend to Fig. 2B. (B) The recombinant CN2 was incubated with EDTA to remove metal ions. After dialysis, 10 μM $ZnCl_2$, $CuCl_2$, $MgCl_2$ or $MnCl_2$ was added to the enzyme, and carnosinase activities were measured by the method described in the legend to Fig. 2B.

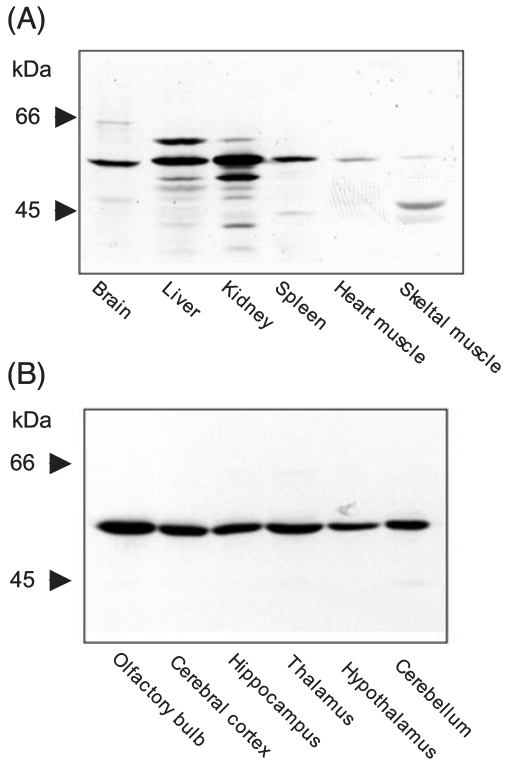

Fig. 4. **Tissue distribution of CN2.** (A) Tissues obtained from male BALB/c mice were homogenized, centrifuged, and the resultant supernatants were analyzed by western blotting with anti-CN2 antibodies. Tissue extracts containing 10 mg protein were applied to each lane. (B) Mouse brain extracts (10 mg protein) were analyzed by western blotting with anti-CN2 antibodies.

that, only Mn^{2+} among the metal ions tested could restore the enzymatic activity of CN2. In contrast, Zn^{2+}, Cu^{2+} and Mg^{2+} had no effect on the activity.

To examine the tissue distribution of the CN2 protein, we raised antiserum against CN2 in a rabbit and affinity purified an antibody with a resin coupled with the recombinant CN2 protein. Western blot analysis showed that the antibody reacted with a 52 kDa protein in tissues including the brain, liver, kidney and spleen, with the highest expression in the kidney (Fig. 4A). The molecular weight was consistent with the value calculated from its cDNA sequence. There were a few additional bands that reacted with the antibody in the kidney and liver extracts, indicating that several variants of CN2 might be expressed in specific tissues. In the brain, the protein was present in all brain areas tested including the olfactory bulb, cerebral cortex, hippocampus and hypothalamus (Fig. 4B). In contrast, little or no signals were found in extracts of skeletal and heart muscle.

The distribution of CN2 in rat brain was further investigated by immunohistochemistry using affinity-purified anti-CN2 antibodies. In the brain, CN2 was expressed in all brain regions, consistent with the result of western blotting, but its expression was extremely high in several neuronal populations. Among the brain regions tested, the highest immunoreactivity was found in the Parafascicular nucleus (pf) of the thalamus (Fig. 5A). This immunoreactivity was likely to be due to the specific binding of antibodies to CN2 because these signals were almost completely abolished when the antibody was preincubated with purified recombinant CN2 (Fig. 5B). A higher magnification image showed that strong immunoreactivity was found in the neuronal cell bodies and neurites in the pf (Fig. 5C). On the other hand, neurons in the adjacent areas did not show strong immunoreactivity.

High immunoreactivity to the anti-CN2 antibody was also found in several other brain areas (Fig. 6). In the olfactory bulb, immunoreactivity was fond in the neuronal cell bodies and neurites of the mitral cell layer (Fig. 6A). In the hypothalamus, strong immunoreactivity was found in the tuberomammillary nucleus (Fig. 6B) and also in several neurons of the cerebral cortex (Fig. 6C). In contrast, in the striata, which receives projections from pf, a higher level of immunoreactivity was observed in neuronal processes than in cell bodies (Fig. 6D).

To determine whether the expression of CN2 in glial cells was observed at comparable levels to that in neurons, we tried double staining of brain sections with anti–glial fibrillary acidic protein (GFAP) and anti-CN2 antibodies. As shown in Fig. 7, GFAP-positive cells were

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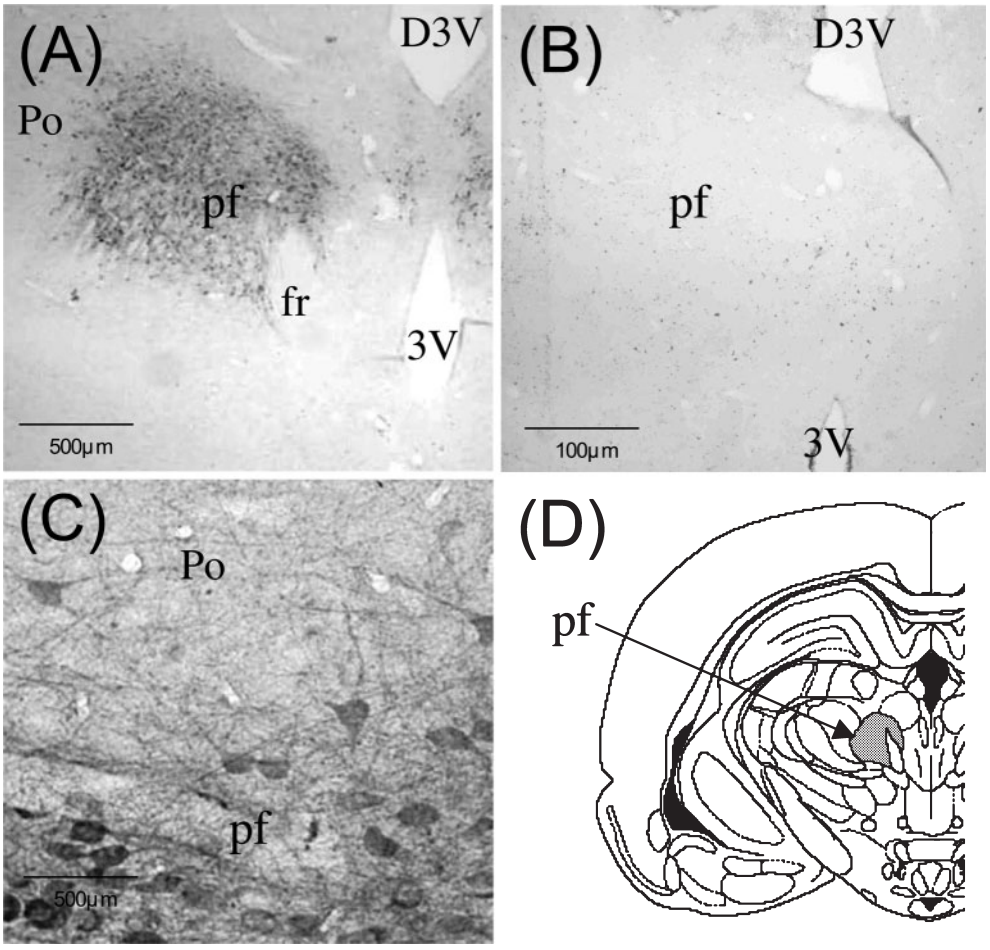

Fig. 5. **Immunohistochemical localization of CN2 in the thalamus.** A rat brain was fixed by perfusion with 4% paraformaldehyde and coronal frozen sections were prepared. The sections were then stained with the affinity-purified anti-CN2 polyclonal antibody and visualized by the ABC detection system. (A) A photomicrograph showing anti-CN2 immunoreactivity in the thalamus containing the parafascicular nucleus (pf). (B) The primary antibody was preincubated with purified recombinant antigen before immunohistochemical staining of the same area as shown in (A). (C) A higher magnification image of the same section as that shown in (A). (D) A brain atlas showing the parafascicular nucleus in which CN2 is highly enriched (arrow). pf, parafascicular nucleus; 3V, the third cerebral ventricle; D3V, the dorsal part of the third cerebral ventricle; Po, Posterior thalamic nucleus.

observed in sections of the olfactory bulb and pf, but none of these cells showed immunoreactivity with CN2. There were weak yellow signals in the merged image of the olfactory bulb. But the signals were so weak that it was not clear whether they indicate the presence of astrocytes expressing CN2.

DISCUSSION

The purpose of the present study was to identify a protein that has carnosinase activity in mammals and clarify its physiological functions in the brain. To find such a protein, we searched for a cDNA that shares sequence similarity with pepV, a L-carnosine digesting enzyme originally found in *Lactobacillus delbrueckii* (*10*) and later in *Lactococcus lactis* (*22*). It belongs to a member of the metallopeptidase M20 family (*9*), which includes enzymes of various species from bacteria to mammals, including disuccinyldiaminopimelate desuccinylase (dapE) (*16*), aminoacylase (acy1) (*17*, *18*), acetylornitine deacetylase (argE) (*19*) and carboxypeptidase G2 (*20*). All these enzymes have activities to hydrolyze the peptidic bonds of small molecules. Since this family contains functionally related proteins of distant species, we postulated that some mammalian proteins with similar activity could be found in this family.

Using the psy-Blast search program, we found a mouse cDNA (NM_023149) that shares 17% sequence identity with pepV. It was originally isolated by the RIKEN cDNA project for which a large number of full length cDNAs have been collected (*14*). At that time, we found no other cDNA entries with sequence similarity, but later found two other cDNAs with sequence similarity, human glutamate carboxypeptidase–like protein 1 and 2, in the NCBI database. An alignment of these proteins showed NM-023149 to be closely related to human glutamate carboxypeptidase-like protein 1 with a sequence identity of 99%, suggesting that it may represent the human counterpart of NM_023149. On the other hand, human glutamate carboxypeptidase-like protein 2 showed 30% sequence identity with NM_023149 with an additional N-terminal protrusion that seems to be the signal peptide for secretion. This indicates that glutamate carboxypeptidase–like protein 2 is a secretory type of enzyme closely related to NM_023149.

To confirm that the protein product was an active enzyme, the recombinant full length protein product of NM_023149 was purified and its catalytic activity was tested using various substrates. We found that the recombinant protein actually had L-carnosine degrading activity to yield L-histidine and β-alanine. In addition, it also degraded some other peptides including anserine, Gly-Phe, and Gly-Gly. In contrast it did not degrade homocarnosine and a tripeptide, Gly-Gly-Gly. These results suggest that the protein digests a wide range of peptides including L-carnosine.

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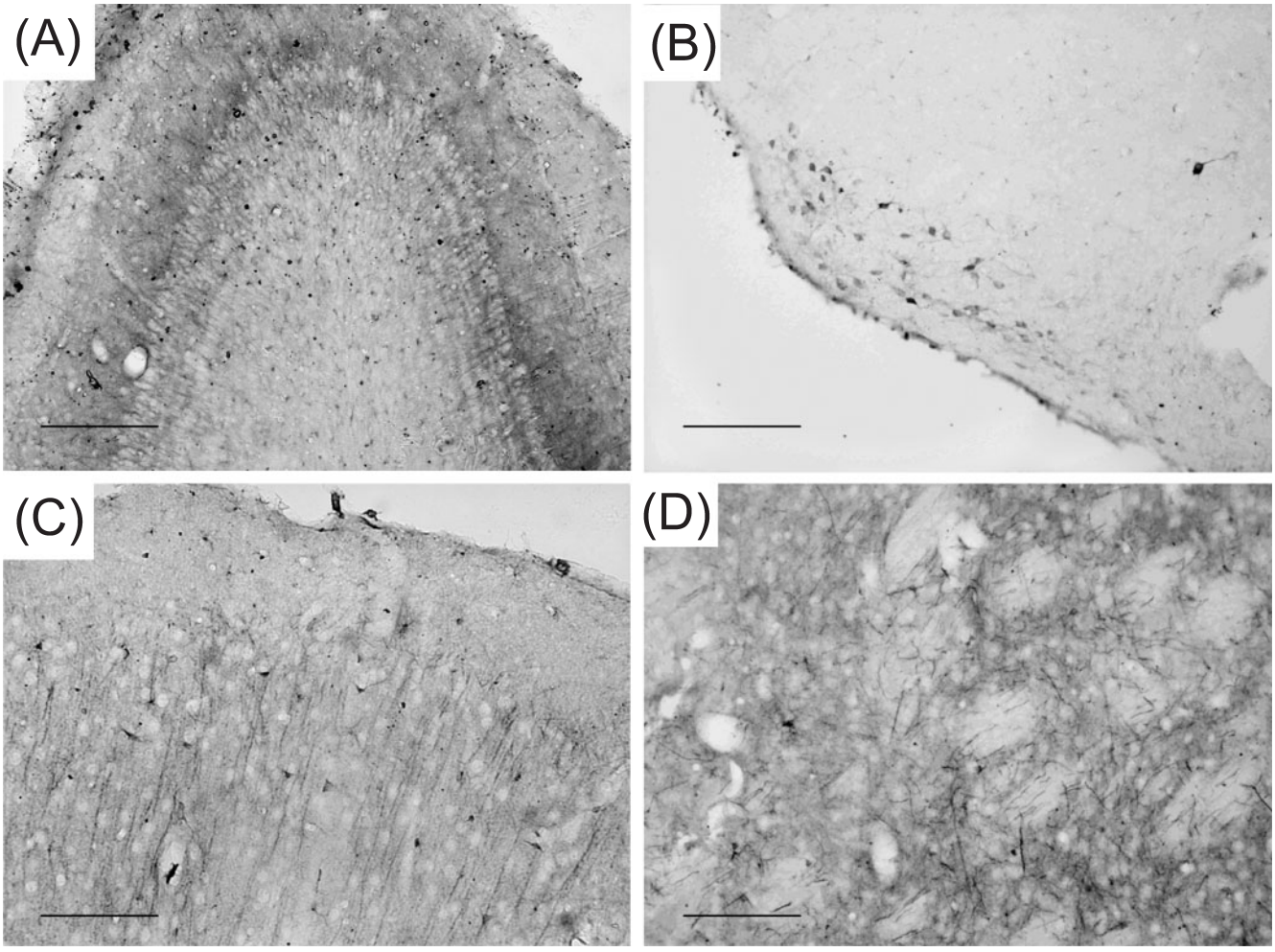

Fig. 6. **Immunohistochemical localization of CN2 in various brain regions.** Coronal frozen sections of rat brain were stained with an anti-CN2 antibody as described in the legend to Fig. 5. (A) olfactory bulb, (B) ventral mammillary nucleus, (C) cerebral cortex, (D) striatum. Bar indicates 500 mm.

During the preparation of this paper, two types of carnosinases, designated as CN1 and CN2, have been reported (*11*). CN1 is a secretary protein with a relatively high specificity for L-carnosine, while CN2 is a cytosolic protein with a wide spectrum of substrate specificity. A sequence alignment showed that the open reading frame of NM_023149 is identical to CN2, and that glutamate carboxylase-like protein 2 is identical to CN1. CN2 has been shown to have a wide specificity for dipeptides and its activity is sensitive to bestatin. These characteristics of CN2 are consistent with our observations of the protein product of NM_023149, and thus we call this protein CN2 hereafter.

CN2 is structurally related to metalloproteinases that require divalent cations for their enzymatic reaction, and has potential metal binding sites as judged by sequence alignment with peptidase M20 family members. This was confirmed by the fact that CN2 is inactive in the presence of EDTA. In addition, its activity was recovered by the addition of Mn^{2+}, suggesting that Mn^{2+} is an effective divalent cation for its activity. In contrast, Zn^{2+}, Cu^{2+}, and Mg^{2+} did not stimulate activity. These results suggest that CN2 is a metalloenzyme that requires Mn^{2+} for its catalytic activity. This is not always the case with other M20 family proteins such as carboxypeptidase G2, which has Zn^{2+} in its active site (*23*). The structural basis for the metal selectivity, as well as substrate specificity, will be clarified by elucidating its three-dimensional structure.

In order to elucidate the physiological function of CN2 in the brain, we raised an antibody against CN2 to examine its tissue distribution. Western blot analysis showed that the antibody reacted with a single 52 kDa band in brain extracts, indicating that the antibody specifically recognized CN2. It was expressed in all brain areas tested at similar levels when examined by western blot analysis, but a more detailed immunohistochemical staining analysis showed that a few neuronal populations express much higher levels of CN2 than most other neurons. First, it was found to be highly expressed in the thalamic parafascicular nucleus in which both neuronal cell bodies and neurites were stained. In addition, neuronal fibers, but not neuronal cell bodies, of the striatum showed strong immunoreactivity. Striatum is one of the major targets of the glutamatergic projection from the parafascicular nucleus, indicating that CN2-positive fibers in the striatum originate from the parafascicular

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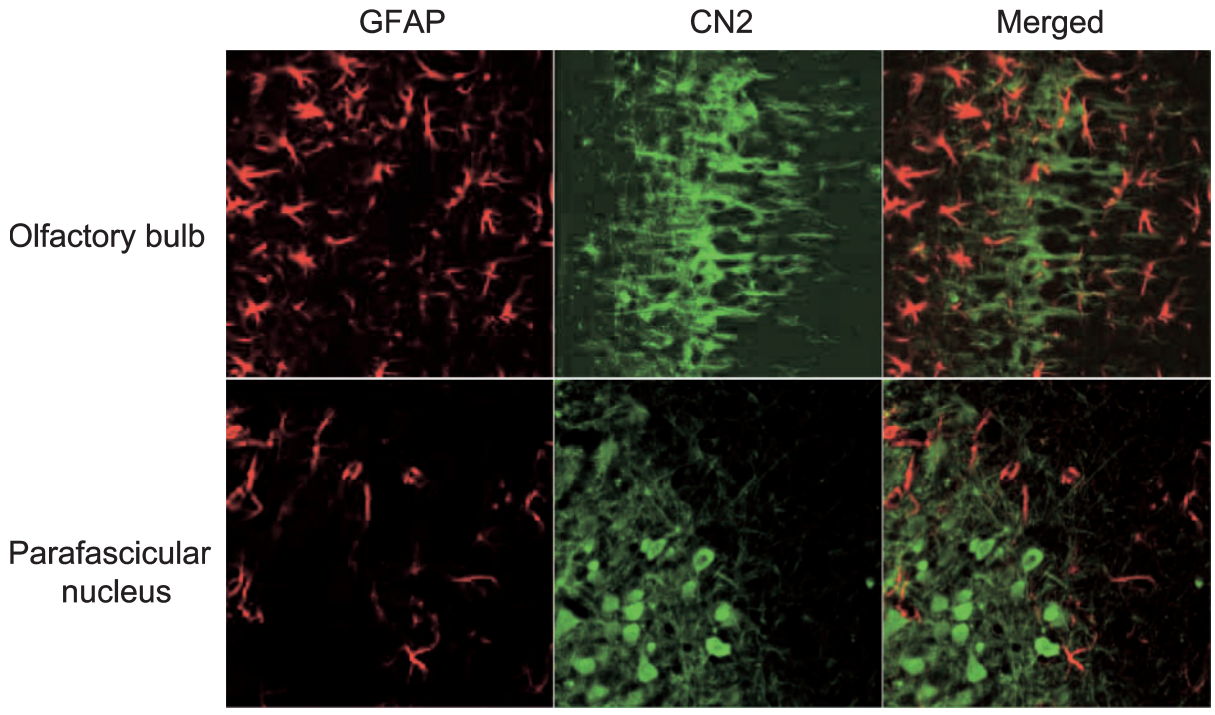

Fig. 7. **Double staining of brain sections with anti-GFAP and anti-CN2 antibodies.** Coronal frozen sections of rat brain were incubated with anti-GFAP (red) and the anti-CN2 (green) antibodies, and then with rhodamine-labeled anti-mouse IgG and fluorescein-labeled anti-rabbit IgG. Fluorescence images were obtained on a confocal scanning microscope. (upper panels) olfactory bulb, (lower panels) parafascicular nucleus of the thalamus.

nucleus. These neurons form an ascending system from the brainstem to the cerebral cortex, and thus CN2 might be involved in this glutamatergic pathway.

Second, CN2 was found to be highly expressed in the mitral cell layer of the olfactory bulb. L-Carnosine is known to be present in the olfactory epithelium and olfactory bulb (*24*), and has been implicated in the neuronal transmission of odor stimulations to the brain. Therefore, the present results suggest that one possible function of CN2 is to eliminate L-carnosine from the nerve terminals of olfactory neurons.

Third, a higher level of CN2 was found in neurons of the hypothalamic tuberomammillary nucleus of the hypothalamus. This is the nucleus where histamine-containing neurons are located as demonstrated by immunohistochemistry with anti-histidine decarboxylase antibody, an enzyme required for histamine synthesis (*25*). From these neurons, histaminergic fibers project to many brain areas including the hypothalamus to regulate a variety of physiological events such as the sleep-wake cycle and energy homeostasis (*26*, *27*). One possible interpretation is that CN2 could supply histidine, the source for histamine biosynthesis by histidine decarboxylase, by degrading L-carnosine and other dipeptides.

Carnosine is present in mammalian brain, especially enriched in several populations of neurons and astrocytes in the central nervous system (*3*). Our previous studies have shown that the injection of carnosine into the brain causes a variety of physiological effects such as lowering the blood glucose levels and regulation of the autonomic nervous system. These findings indicate that carnosine is one of the bioactive peptides acting in neuronal networks in the brain. In addition, we have previously shown that the effect of carnosine is blocked by a histamine H3 antagonist. A possible explanation for these phenomena is that carnosine is active when it is converted to histamine. If this pathway actually exists in brain, carnosinase is an essential enzyme required for the conversion of carnosine to histidine. This idea is consistent with our current observation that CN2 is present in the tuberomammillary nucleus of the hypothalamus where histidine decarboxylase, an enzyme essential for histamine synthesis, is located. Whether or not CN2 colocalizes with histidine decarboxylase in this brain area remains to be examined.

In the present study, we have characterized the molecular structure, enzymatic properties and immunohistochemical localization of a L-carnosine–digesting enzyme. However, since several other enzymes that digest L-carnosine are known to be present in mammalian tissues, other types of enzymes must be examined in the future. In addition, the molecular characteristics of L-carnosine synthase are still largely unknown; thus further studies are required.

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