A new oyster carbonic anhydrase in *Crassostrea gigas*

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Abstract

In metazoans, carbonic anhydrase（CA）is a common enzyme that catalyzes the reversible hydration of carbon dioxide. CA plays important roles in many biological processes, and in molluscs several CA-like proteins are thought to be responsible for calcification in shell formation. As part of our investigation of the process of shell formation, we searched for a CA gene in the oyster *Crassostrea gigas*, with resultant identification of a single cDNA that encodes two CA domains. This atypical CA is similar to an α-CA and is mainly expressed in the egg. This suggests that the newly identified CA is responsible for production of bicarbonate in the early development phase prior to metamorphosis.

Key words: mollusc, shell, calcification, carbonic anhydrase, cDNA

1. Introduction

The current understanding of the increase in mineralized structures during the Cambrian includes the proposal that biological apparatuses enabling a mineralization process such as calcification developed during this period [(1)]. It remains unclear how calcified hard tissues are encoded at the molecular level, particularly with regard to the process of crystallization of calcium carbonate and growth of calcified hard tissues such as shells in molluscs. Information on molecules involved in biomineralization is limited and it is uncertain if toolkits for shell biomineralization are evolutionarily related among metazoans that possess mineralized hard tissues.

Many organisms have developed CO_2-concentrating mechanisms in which inorganic carbon is produced in the form of bicarbonate by the action of carbonic anhydrases（CAs）. CAs are widespread zinc-containing enzymes that catalyze reversible hydration of carbon dioxide to bicarbonate [(2)]. Based on sequence information, CAs are classified into five groups: α（mainly animals）, β（eubacteria）, γ（archea and some eubacteria）, and δ and ζ（marine diatoms）[(3)]. In mammals, at least 16 α-type CAs have been reported [(4, 5)]. In molluscs, several CAs and CA-related molecules have been identified and are thought to have a role in calcification in shell formation [(6−10)]. In this study, we isolated a new CA gene from the oyster *Crassostrea gigas* and characterized its primary structure, with the goal of obtaining more information on the roles of CAs in shell biomineralization.

2. Materials and Methods

2.1 RNA isolation

Tissues were dissected from one specimen of *C. gigas* and washed in artificial seawater. Following

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homogenization in Trizol reagent (Invitrogen), homogenized tissues were subjected to RNA isolation. RNAs were further purified using an RNeasy kit (Qiagen).

2.2 cDNA amplification

Total RNA (10 μg) from the mantle was subjected to first strand cDNA synthesis in a 25 μl reaction containing 50 mM Tris-HCl (pH8.3), 40 mM KCl, 6 mM MgCl2, 10 mM DTT, 0.5 mM dNTP, 20 ng/μl oligo-dT primer, and 8 unit/μl Superscript II reverse transcriptase (Invitrogen) at 42℃ for 2 hours. PCR amplification was carried out using AmpliTaq gold (Applied Biosystems) and primers based on the conserved domains of CAs in mammals and nacrein in pearl oyster: a 5′ primer〔MRMCARTCICCAAT

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   1  ACGCGGGGAATGCAAGCAAGATCATTATTTAAAACACTTCATGTCCCTTTGTGGGCCTCTTTTTCGTCAGGACTGTAAATGCAAGTTGTC
                                                 M  S  L  C  G  P  L  F  R  Q  D  C  K  C  K  L  S    17
  91  AATTTTAGAGGGCCCATATTTAGTATACTGGAAGTTTGCCCTTCAACAAGCAATGTCTGCTGTGCTAGGGATTTTATCTCTTTATCTCAC
       I  L  E  G  P  Y  L  V  Y  W  K  F  A  L  Q  Q  A  M  S  A  V  L  G  I  L  S  L  Y  L  T    47
 181  CTGTGCAGCTGCTGCATCAGGCTCAAATTGGGGATATAAAGGATTAAATGGACCAGAGAATTGGTACAAGCTGTTACACAATGCCTGCTC
       C  A  A  A  A  S  G  S  N  W  G  Y  K  G  L  N  G  P  E  N  W  Y  K  L  L  H  N  A  C  S    77
 271  AGGACTCAGCCAGTCACCGATCAACATAGAGAGGGAGAAGGCCATCTATGACCCCACCCTCAATGACTTCGCCCTCTGGCACGACCCCCC
       G  L  S  Q  S  P  I  N  I  E  R  E  K  A  I  Y  D  P  T  L  N  D  F  A  L  W  H  D  P  P   107
 361  TCGCCCCGGCTCATATTTTGATGTCACAAACAATGGACACACAATCCAAGTTGACACCGTTGGACCATTCTATGTATCCAATGGGGGCTT
       R  P  G  S  Y  F  D  V  T  N  N  G  H  T  I  Q  V  D  T  V  G  P  F  Y  V  S  N  G  G  L   137
 451  GCCTGCAGTATATAGCACCGCCCAGTTTCACTTTCACTGGGGGCACGAAAACGACCACGGGTCAGAGCATTTGATTGATGGGAGGGCCAG
       P  A  V  Y  S  T  A  Q  F  H  F  H  W  G  H  E  N  D  H  G  S  E  H  L  I  D  G  R  A  S   167
 541  TCCGTTAGAGCTCCATGTTGTCAGCTACAATTCGGATGAGTTTGACTTAATCACAGAAGCCATGGTTCAACCTCAGGGATTAGCAGTACT
       P  L  E  L  H  V  V  S  Y  N  S  D  E  F  D  L  I  T  E  A  M  V  Q  P  Q  G  L  A  V  L   197
 631  CGGAGTTATGTATGAAATAGTGGATGAAGATAACCCTGCCTTAGAGCCCATTATCAATGCCATGAACACTGTCAGAGACCCTGGTGCTAA
       G  V  M  Y  E  I  V  D  E  D  N  P  A  L  E  P  I  I  N  A  M  N  T  V  R  D  P  G  A  K   227
 721  AGCTCGAATCGATGCCCAGGCCCTGAGGAACTTCCTCCCCGAGGACACTTCTATGTACTTTAGGTACAACGGCTCACTGACCACACCAGG
       A  R  I  D  A  Q  A  L  R  N  F  L  P  E  D  T  S  M  Y  F  R  Y  N  G  S  L  T  T  P  G   257
 811  CTGTTTTGAGAGTGTCATTTGGACTGTGTTTGATCAGAAACAGACCATTTCCCACAGACAGATGAAGATGTTCCGCACCCTTCTCCAACA
       C  F  E  S  V  I  W  T  V  F  D  Q  K  Q  T  I  S  H  R  Q  M  K  M  F  R  T  L  L  Q  H   287
 901  TAAGAGTAAACAAGGGAGGAAGAAGAGGAGTCTGACTGAGCAGTGTGAACAGGCAGCCGAGGATGTCCTCGGAGAGATAGGAATCAAAGG
       K  S  K  Q  G  R  K  K  R  S  L  T  E  Q  C  E  Q  A  A  E  D  V  L  G  E  I  G  I  K  G   317
 991  CAAGGTCCTTGAGGAGCTGTCTCTGGAACTGCAGATGGAGAAAAGGCAGCAGGAGCAGGCCAAACAACAAGAAAGCACCATCATTAAGGA
       K  V  L  E  E  L  S  L  E  L  Q  M  E  K  R  Q  Q  E  Q  A  K  Q  Q  E  S  T  I  I  K  E   347
1081  ATACAAGATGACGCATGGCATGAGTGAGGATGAGCCGGTCAAGTTGGAGGATATCCCAGCATCCTTCATTCAGGAGGCCCTGTACGATAA
       Y  K  M  T  H  G  M  S  E  D  E  P  V  K  L  E  D  I  P  A  S  F  I  Q  E  A  L  Y  D  N   377
1171  CTTCCGACCTGTACAGCCCCTCAACAGTAGAACCGTGTACTCTTCATTCAAGATTGAGCCCTCCAAAAAACCTGTAAAAAAGATTCCTTG
       F  R  P  V  Q  P  L  N  S  R  T  V  Y  S  S  F  K  I  E  P  S  K  K  P  V  K  K  I  P  W   407
1261  GGGATACAAGGGCAAAAAAGGTCCCTCCAACTGGCATACACTGTCTGAGAACTCTTGTCTGGGAATGTACCAGTCGCCTATCAACATAGA
       G  Y  K  G  K  K  G  P  S  N  W  H  T  L  S  E  N  S  C  L  G  M  Y  Q  S  P  I  N  I  D   437
1351  CAGAGAGATGACCATCTACAACCCAGACATCAACAACTTCATCTTCTGGTACGACCCTCCTGCTCCCAATGCCGAATTCTATGTTTTCAA
       R  E  M  T  I  Y  N  P  D  I  N  N  F  I  F  W  Y  D  P  P  A  P  N  A  E  F  Y  V  F  N   467
1441  CAATGGCCACACAGTACAAGTGAACACAATTGGACCCTACTATGTGGCCAATGGGGGACTGTCTTCCGTCTACAGCACTGCCCAGTTCCA
       N  G  H  T  V  Q  V  N  T  I  G  P  Y  Y  V  A  N  G  G  L  S  S  V  Y  S  T  A  Q  F  H   497
1531  CTTCCACTGGGGAGCAGAGAATAGCTTTGGCTCAGAACACCAGATTGATGGACAGAGTTTCCCTCTTGAACTTCATGTGGTGAACTATGA
       F  H  W  G  A  E  N  S  F  G  S  E  H  Q  I  D  G  Q  S  F  P  L  E  L  H  V  V  N  Y  D   527
1621  CTCAGTGAACTATGCTTCCATCAGTCAGGCTATGACTGAGCCTGGTGGTTTGGCTGTCCTGGGTGTTCTCTTCAGGGTAGGTGATGAGGA
       S  V  N  Y  A  S  I  S  Q  A  M  T  E  P  G  G  L  A  V  L  G  V  L  F  R  V  G  D  E  D   557
1711  TAACCAAGCCTTAGAACCAATAATTAATGCCATGAAAGCTGTCCAAGATCCAGAGGATCATTCTAAGGTGAAGATTCCAGCCCAGGCGAT
       N  Q  A  L  E  P  I  I  N  A  M  K  A  V  Q  D  P  E  D  H  S  K  V  K  I  P  A  Q  A  I   587
1801  CAAAAACTTCCTGCCTGAGGACACCACTAAGTTCTATAGGTACAACGGTTCACTGACCACGCCCGGATGTTTCGAGAGCGTCATTTGGAC
       K  N  F  L  P  E  D  T  T  K  F  Y  R  Y  N  G  S  L  T  T  P  G  C  F  E  S  V  I  W  T   617
1891  TGTGTTTGAGGACAAACAGACTTTATCTCACAGACAGATGGAAGAATTCCGTAAACTTTTACAACATCGAGTTGAAAAGGGGGAAATGAA
       V  F  E  D  K  Q  T  L  S  H  R  Q  M  E  E  F  R  K  L  L  Q  H  R  V  E  K  G  E  M  K   647
1981  GAAACTGGATTTGTCTGGTGGACAGCGGAAGGCAGCTAAGGAGATCTTGGTGGAGGCTGGTATAATCGGGGACTCAAAACAAGAACGGGA
       K  L  D  L  S  G  G  Q  R  K  A  A  K  E  I  L  V  E  A  G  I  I  G  D  S  K  Q  E  R  E   677
2071  GCTCACACAGGCACTCAGGAAGAAGAAGGGTTTTCCCCAAATGATGCAAGATCAACCAGCATTAGATGAAGAGATTAAGGCCAACGTTAC
       L  T  Q  A  L  R  K  K  K  G  F  P  Q  M  M  Q  D  Q  P  A  L  D  E  E  I  K  A  N  V  T   707
2161  AGTAGAGGAGATCACTATAGAAATTGTTCAGGAACAACTGGTGAACAACTACAGACCTGTTCAACCAATCAACAACAGGCTCATCTACCG
       V  E  E  I  T  I  E  I  V  Q  E  Q  L  V  N  N  Y  R  P  V  Q  P  I  N  N  R  L  I  Y  R   737
2251  AACATTCACGTACGAACCAAAGAAGGAAGTTCGTTACGTTGTACGCTACGATTCACAAAATAAGAACCTCGCTATTTCATCATCAGTGCC
       T  F  T  Y  E  P  K  K  E  V  R  Y  V  V  R  Y  D  S  Q  N  K  N  L  A  I  S  S  S  V  P   767
2341  ATCTTTTATTGTTTTATTAACATGCGTCATAATTTCATTGTTCTTTAGAAAGTAAGATAATTATTATAGTTTTATTCTTAAC
       S  F  I  V  L  L  T  C  V  I  I  S  L  F  F  R  K  *                                      784
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Fig. 1. Nucleotide and deduced amino acid sequences of the *C. gigas* CA. DDBJ/EMBL/GenBank accession number: AB794884.

corresponding to RQSP〕and a 3′ primer〔TRTGYTCITGNCC corresponding to GSEHS〕. The PCR amplification product was ligated into the pGEM-T easy TA cloning vector（Promega). To obtain the 5′- and 3′-ends of the oyster CA, RACE were performed according to the manufacturer's instructions (Takara Bio) using 2 μg of mantle poly (A) + RNA and specific primers: an antisense primer 5′-CATTGAGGGTGGGGTCATAGATGGC-3′ for 5′-RACE and a sense primer 5′-CTGCAGTATATAGCACCGCCCAGTT -3′ for 3′-RACE. Both ends were cloned described above.

2.3 Sequence analysis

Plasmids containing the oyster CA cDNAs were purified from *Escherichia coli* and used in a dye-terminator reaction with a Big-Dye terminator kit（Applied Biosystems). Each sequence was assembled and translated into an amino acid sequence.

2.4 Northern blot analysis

Total RNA was separated by electrophoresis in a 1% agarose gel containing formaldehyde, and then transferred to a Hybond-N filter（GE Healthcare). After hybridization at 65℃ , the filter was washed in 0.5 × SSC at 65℃ and then subjected to signal detection by BAS2500 Image analyzer（Fuji film).

B

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GSNWGYKGLNGPENWYKLLHNACSGLSQSPINIEREKAIYDPTLNDFALWHDPPRPGSYF 113
KIPWGYKGKKGPSNWHTLSENSCLGMYQSPINIDREMTIYNPDINNFIFWYDPPAPNAEF 463

DVTNNGHTIQVDTVGPFYVSNGGLPAVYSTAQFHFHWGHENDHGSEHLIDGRASPLELHV 173
YVFNNGHTVQVNTIGPYYVANGGLSSVYSTAQFHFHWGAENSFGSEHQIDGQSFPLELHV 523

VSYNSDEFDLITEAMVQPQGLAVLGVMYEIVDEDNPALEPIINAMNTVRDP-G-AKARID 231
VNYDSVNYASISQAMTEPGGLAVLGVLFRVGDEDNQALEPIINAMKAVQDPEDHSKVKIP 583

AQALRNFLPEDTSMYFRYNGSLTTPGCFESVIWTVFDQKQTISHRQMKMFRTLL       284
AQAIKNFLPEDTTKFYRYNGSLTTPGCFESVIWTVFEDKQTLSHRQMEEFRKL       636
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Fig. 2. Schematic representation of the *C. gigas* CA（A）and comparison of the two CA domains（B). A dash indicates a gap added to the sequences.

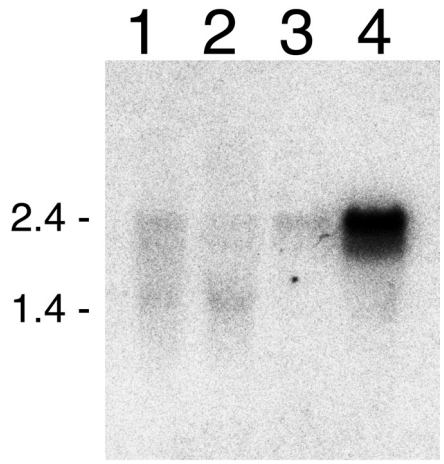

Fig. 3. Expression of the CA gene in *C. gigas*. Total RNAs prepared from different tissues were hybridized with the CA probe. Lane 1, gill; lane 2, mid-gut; lane3, mantle; lane4, egg. RNA quality was checked in staining with ethidium bromide (data not shown). Molecular size standards of RNA are shown in kb.

3. Results and Discussion

RT-PCR amplification using the mantle RNA of *C. gigas* permitted identification of a cDNA fragment(246 bp) with similarity to known CAs. To obtain the entire sequence of the oyster CA, we performed 5′- and 3′-RACE using the determined cDNA sequence as a source of primers. The nucleotide and amino acid sequences of the *C. gigas* CA are shown in Figure 1. The cDNA is 2422 bp in length and has an open reading frame of 2352 nucleotides coding for a protein of 784 amino acids. The predicted protein is composed of two CA domains (Fig. 2A) that are highly conserved (Fig. 2B) and show 20-40% identity to α-CAs of metazoans. Since the two CA domains retain critical conserved histidine residues in the zinc binding site, this protein is likely to have CA enzymatic activity. To determine the role of the oyster CA, expression levels of the CA gene were investigated by northern blot analysis. The results showed that the oyster CA is mainly expressed in the egg. In most conchiferans, shells are formed prior to metamorphosis [11], and in bivalves, shells are found at least at the stage of D-shape larvae, which have calcified structures. Therefore, it is possible that the newly identified atypical CA is involved in early calcification prior to metamorphosis in the oyster.

4. References

1. Knoll, A.H. (2003) Biomineralization and Evolutionary History. Rev. Mineral. Geochem. 54, 329-356.
2. Tashian, R.E. (1989) The carbonic anhydrases: Widening perspectives on their evolution, expression and function. BioEssays 10, 186-192.
3. Zimmerman, S.A. and Ferry, J.G. (2008) The beta and gamma classes of carbonic anhydrase. Curr. Pharm. Des. 14, 716-721.
4. Hewett-Emmett, D. (2000) Evolution and distribution of the carbonic anhydrase gene families. In Chegwidden, W.R., Carter, N.D. and Edwards, Y.H. (eds.), The Carbonic Anhydrases. New Horizons.

Birkhauser, Basel, pp. 29-76.

5．Supuran, C.T.（2008）Carbonic anhydrases--an overview. Curr. Pharm. Des. 14, 603-614.

6．Jackson, D.J., McDougall, C., Woodcroft, B., Moase, P., Rose, R.A., Kube, M., Reinhardt, R., Rokhsar, D.S., Montagnani, C., Joubert, C., Piquemal, D. and Degnan, B.M.（2010）Parallel evolution of nacre building gene sets in molluscs. Mol. Biol. Evol. 27, 591-608.

7．Kono, M., Hayashi, N. and Samata, T.（2000）Molecular mechanism of the nacreous layer formation in *Pinctada maxima*. Biochem. Biophys. Res. Commun. 269, 213-218.

8．Miyamoto, H., Miyashita, T., Okushima, M., Nakano, S., Morita, T. and Matsushiro, A.（1996）A carbonic anhydrase from the nacreous layer in oyster pearls. Proc. Natl. Acad. Sci. USA 93, 9657-9660.

9．Miyamoto, H., Miyoshi, F. and Kohno, J.（2005）The carbonic anhydrase domain protein nacrein is expressed in the epithelial cells of the mantle and acts as a negative regulator in calcification in the mollusc *Pinctada fucata*. Zool. Sci. 22, 311-315.

10. Miyamoto, H., Yano, M. and Miyashita, T.（2003）Similarities in the structure of nacrein, the shell-matrix protein, in a bivalve and a gastropod. J. Moll. Stud. 69, 87-89.

11. Eyster, L.（1986）Shell inorganic composition and onset of shell mineralization during bivalve and gastropod embryogenesis. Biol. Bull. 170, 211-231.

和 文 抄 録

マガキの新規炭酸脱水酵素

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炭酸脱水酵素は後生動物に広く存在しており、二酸化炭素の水和を可逆的に触媒する。機能的には様々な生命過程で重要な役割を担っており、軟体動物では炭酸脱水酵素や類似タンパク質が貝殻形成における石灰化に関与していることが示唆されている。本論文では、貝殻形成の過程を調べる研究の中で同定したマガキの新規炭酸脱水酵素について報告する。単離した cDNA は、二つの CA ドメインを有しており、典型的な炭酸脱水酵素とは構造が異なっていたが、それぞれの CA ドメインは α タイプの炭酸脱水酵素との類似性を示した。また、今回同定した新規炭酸脱水酵素は卵での発現が著しく高く、変態前の重炭酸イオンの生成を担っていると推察される。

キーワード：軟体動物、貝殻、石灰化、炭酸脱水酵素、cDNA

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