Cloning and Expression of *Brassica napus* β-Carbonic Anhydrase cDNA

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Z. Naturforsch. **64c**, 875–881 (2009); received July 9, 2009

A new full-length β-carbonic anhydrase cDNA was obtained from *Brassica napus* by homologous cloning. The cDNA has an open-reading frame of 996 nucleotides, encoding 331 amino acids with a calculated molecular weight of 35,692 Da and an estimated pI value of 5.459. The deduced amino acid sequence of β-carbonic anhydrase from *Brassica napus* shared significant identity with β-carbonic anhydrases from *Brassica carinata*, *Arabidopsis thaliana*, and *Thlaspi caerulescens* (97.9%, 94%, and 93.5% identity, respectively). This cDNA was expressed in *Escherichia coli* BL21 (DE3) using the expression vector pET-32a(+). The expression band corresponded to the calculated mass plus the *N*-terminal fusion protein derived from the vector.

Key words: Brassica napus, β-Carbonic Anhydrase, Expression in *E. coli*

Introduction

Carbonic anhydrase (CA, carbonate dehydratase, carbonate hydrolyase; EC 4.2.1.1) is a zinc-containing enzyme that catalyzes the reversible hydration of CO_2 ($CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$) (Khalifah, 1971). CA is one of the enzymes whose catalytic velocities are confirmed to be the fastest. It is widely distributed in animals, plants, archaebacteria and eubacteria (Hewett-Emmett and Tashian, 1996). The known CAs can be grouped into five independent families (α, β, γ, δ, and ε), which have no primary sequence similarities and seem to have evolved independently (Hewett-Emmett and Tashian, 1996; Roberts *et al.*, 1997; So *et al.*, 2004). Plants have four types of CAs (α, β, γ, and δ) which are involved in various biological processes including photosynthetic CO_2 fixation, respiration, biosynthesis, CO_2 transfer, pH regulation, and ion exchange (Badger and Price, 1994; Moroney *et al.*, 2001; Smith and Ferry, 2000). β-CA has been most intensively studied in higher plants because of the critical role it plays in photosynthesis. The native molecular weights of CAs range from 140 to 250 kDa, with a subunit mass of 26 to 34 kDa. The molecule has been shown to bind one zinc molecule per subunit (Reed and Graham, 1981).

Oilseed rape is an annual herbaceous C_3 plant of *Brassica* (Brassicaceae). It is an important oil crop planted extensively in the world. At present, the planting area and yield of rape in China account for one third in the world (Fu, 2000). Studies focus on how to increase the yield and stress resistance of rape nowadays. CA is very important in many physiological functions that involve carboxylation and decarboxylation reactions, including both photosynthesis and respiration. The photosyntheic rate, CA activity and crop yield are closely related with each other. In C_3 plants, β-CA is a major component of leaf protein (0.5–2% of the total) and only two CAs have been localized, one in chloroplast stroma and one in the cytoplasm (Badger and Price, 1994; Moroney *et al.*, 2001). However, little is known about CAs in *B. napus*, and cloning of the β-CA gene in *B. napus* hasn't been reported until now. Therefore, this is the first time for us to report the cloning and expression of the β-CA gene in *B. napus*.

In this work, we isolated a full-length β-CA cDNA from *B. napus* by a homology-based PCR cloning strategy. The cDNA encoding β-CA was successfully expressed in *Escherichia coli* cells as a His fusion protein. The procedure of establish-

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0939–5075/2009/1100–0875 $ 06.00

ing the expression of a recombinant $(His)_6$-CA fusion protein in *E. coli* is presented.

Material and Methods

Bacterial strains and plasmids

E. coli DH5 α (Takara, Dalian, China) and *E. coli* BL21 (DE3) (Novagen, Darmstadt, Germany) were used as the hosts for plasmid amplification and gene expression, respectively. Plasmid pMD 18-T simple vector (Takara, Japan) and pET-32a(+) (Novagen) were used as vectors for cloning and expression, respectively.

Cloning and sequence analysis of β-CA

Total RNA was isolated from leaves of *B. napus* using the Trizol kit (Invitrogen, Carlsbad, USA). First-strand cDNA was synthesized with a first-strand synthesis kit (Invitrogen) and used for reverse-transcription polymerase chain reactions (RT-PCR). The two PCR primers were designed based on the conserved nucleotide sequences of β-CA from other Brassicaceae plants (*i.e. Arabidopsis thaliana* and *Thlaspi caerulescens*): β-CA F1, 5′-ATGTCGACCGCTCCTCTCTCCGGC-3′; β-CA R1, 5′-TCATACAGAGCTAGTTTCGGAGAGG-3′. DNA amplification was performed using KOD DNA polymerase (Invitrogen) under the following conditions: 94 °C for 4 min, 35 cycles at 94 °C for 45 s, 55 °C for 45 s and 72 °C for 1 min, and, finally, 72 °C for 7 min. The resulting 996-bp amplicon was separated by agarose gel electrophoresis, and the product was extracted from the gel, cloned into the pMD18-T simple vector, and transformed into *E. coli* DH5 α cells. Recombinant clones were PCR-amplified from plasmid DNA which was prepared from several individual transformants. Positive clones were fully sequenced. The sequence result revealed that this gene fragment is a full-length open-reading frame (ORF) of the *B. napus* β-CA gene.

Sequence homologous alignment and similarity search were carried out by BLAST biological software (http://www.ncbi.nlm.nih.gov/blast). ORF analysis and multi-alignment were carried out by DNAMAN 4.0 and Vector NTI suite 9.0. The phylogenetic tree was constructed based on the neighbour joining methods using Mega 4.0.

The nucleotide sequence reported in the present work has been deposited in the GenBank database under the accession number GQ356780.

Construction of the expression vector pET32-CA

RT-PCR was performed using the following specific primers: β-CA F2, 5′-CGCGGATCCATGTCGACCGCTCCTCTCTCCGGC-3′, and β-CA R2, 5′-CCGCTCGAGTCATACAGAGCTAGTTTCGGAGAGG-3′, to obtain the ORF of the β-CA gene. In order to facilitate subcloning of the ORF into the pET-32a(+) vector, the restriction sites of *Bam*H I and *Xho* I were separately introduced into the forward and reverse primers. The PCR products and the pET-32a(+) vector were digested with *Bam*H I and *Xho* I, purified, and ligated, resulting in the plasmid pET32-CA. The resultant plasmid was transformed into *E. coli* DH5 α. The ampicillin-resistant transformants were screened by colony PCR. The recombinant plasmid was extracted and digested with both *Bam*H I and *Xho* I for confirmation. The sequence of the insert was further confirmed by DNA sequencing.

Bacterial expression

The expression plasmid pET32-CA was transformed into the competent *E. coli* BL21 (DE3). The recombinant β-CA was expressed in transformed cells according to the manufacturer's instructions (Novagen). A single positive bacterial colony was inoculated into 3 ml liquid LB medium containing ampicillin (100 μg/ml) and grown overnight. The entire 3 ml seed culture were added to 100 ml LB medium containing ampicillin (100 μg/ml). The mixture was grown at 28 °C to $OD_{600\,nm}$ = 0.7–0.8. Just prior to induction, the 100-ml culture was split into two 50-ml cultures. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to one of the 50-ml cultures to a final concentration of 1 mM and the other culture was used as an uninduced control. After 5 h of incubation at 28 °C, 1 ml culture was harvested by centrifugation, and the pellet was resuspended completely by mixing in 100 μl of 1× phosphate-buffered saline (PBS). 100 μl of 2× SDS loading buffer were added and sonicated. The sample was immediately heated for 3 min at 85 °C to denature the proteins and was then analyzed by SDS-PAGE.

Polyacrylamide gel electrophoresis (PAGE)

The expression of the interesting enzyme was determined by SDS-PAGE. SDS-PAGE was conducted on a 12% (v/w) polyacrylamide gel according to the method of Laemmli (1970). The low

molecular weight standard from MBI Fermentas (Hanover, USA) was used. Protein bands were stained with Coomassie brilliant blue.

Results and Discussion

Cloning and nucleotide sequence analysis of the β-CA gene from B. napus

To clone β-CA genes from *B. napus*, the homologue-based PCR cloning strategy was applied as mentioned above. The two primers were designed based on the conserved sequences of β-CA cDNA from *A. thaliana* (NM_180160) and *T. caerulescens* (AY551530), thus the sequence we wanted to amplify (from ATG to TGA) spanned the ORF. A cDNA fragment was amplified by PCR using the two primers. The resultant cDNA contained a full-length ORF of 996 nucleotides. The ORF encoded a polypeptide of 331 amino acids with a calculated molecular weight of 35,692 Da, consistent with that of other monomeric β-CAs (26–34 kDa) in plants, and an estimated pI value of 5.459 by DNAMAN analysis software. The complete nucleotide sequence and the putative amino acid sequence are shown in Fig. 1. Chloroplastic β-CAs are generally nuclear encoded and so are initially expressed with a two-component signal presequence of about 100 amino acids to direct them to the chloroplast's stroma (Johansson and Forsman, 1992). The deduced amino acid sequence appears to encode an *N*-terminal targeting sequence, which indicated that it is a premature β-CA in *B. napus*.

BLAST search revealed that nobody had reported the cDNA sequence of β-CA from *B. napus*. Therefore, it was the first time for us to report the β-CA gene from *B. napus*. β-CA families have two typical motifs: motif 1 (C-[SA]-D-S-R-[LIVM]-x-[AP]) and motif 2 ([EQ]-[YF]-A-[LIVM]-x(2)-[LIVM]-x(4)-[LIVMF](3)-x-G-H-x(2)-C-G), where x denotes any amino acid (Hulo *et al.*, 2006; Sigrist *et al.*, 2002). There were two distinct groups of β-CA ('Cab'-type and 'plant'-type), differing in their pattern of sequence conservation, active site design and possibly also in their mechanism (Smith and Ferry, 1999; Kimber and Pai, 2000). 'Plant'-type β-CAs observe the principle of two motifs strictly. For example, alignment of β-CAs in nine plants showed motifs 1 and 2: C-S-D-S-R-V-C-P (corresponding to 162aa–169aa in *B. napus*) and E-Y-A-V-L-H-L-K-V-E-N-I-V-V-I-G-H-S-A-C-G (corresponding to 206aa–226aa in *B. napus*). The sequence corresponding to 156aa–226aa in *B. napus* was the highly conserved domain in β-CA families, and motifs 1 and 2 were within the domain. However, alignment of the putative amino acid sequence of β-CA from *B. napus* with that of β-CAs from other plants, generally, showed a rather low overall homology of 40.4%. Sequence analysis by Vector NTI suite 9.0 software showed that five residues were highly conserved in different plant species. The five residues were Asp, Arg, and three amino acids binding zinc ions (Cys, Cys, His), which was consistent with the results of Smith *et al.* (2000). All data are shown in Fig. 2.

The phylogenetic tree was constructed based on the amino acid sequences of β-CAs from *B. napus* and other plants by the neighbour joining (NJ) method using the software Mega 4.0 (Fig. 3). The results showed that β-CA from *B. napus* was highly similar to β-CAs from *A. thaliana* (NP_850491) and *T. caerulescens* (AAS65454) with 94% and 93.5% identity, respectively. Comparison with β-CA from *B. carinata* revealed an even closer relationship (97.9% identity). The minor differences between *B. napus* and *B. carinata* are likely attributable to the species differences within the genus.

Expression of β-CA from B. napus

The amplified β-CA ORF (996 bp) from *B. napus* was inserted into the pET-32a(+) vector, resulting an expression vector for β-CA. The positive recombinant plasmids (pET32-CA) were transformed into competent *E. coli* BL21 (DE3) cells. With the induction of IPTG (1 mM), β-CA was expressed as a fusion protein to $(His)_6$-tag. The *E. coli* cells harbouring the recombinant plasmid were analyzed for expression of the fusion protein on SDS-PAGE (Fig. 4). A protein with an apparent molecular weight of 37.5 kDa was expressed after induction from 5 to 10 h, which corresponded to the size of the fusion protein which had a calculated molecular weight of 37.5 kDa. The SDS-PAGE results showed that the β-CA cDNA (996 bp) from *B. napus* could be normally translated into a complete $(His)_6$-fusion protein as we predicted. The enzyme was expressed in *E. coli* cells as a fusion protein with the 6× His-tag at the *N*-terminus and could be purified by nickel-affinity chromatography. The purified His-tagged fusion protein would be used for further enzymatic characterization.

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1       ATGTCGACCGCTCCTCTCTCCGGCTTCTTCCTCACCTCTCTTTCTCCTTCTCAGCCTTCT
1        M  S  T  A  P  L  S  G  F  F  L  T  S  L  S  P  S  Q  P  S

61      CTCCAAAAACAGACTCTTCGTTCTTCTCCCACCGTGGCTTGCCTTCCCTCATCCTCTTCT
21       L  Q  K  Q  T  L  R  S  S  P  T  V  A  C  L  P  S  S  S  S

121     TCCTCCTCCTCCTCTCGTTCCGTTCCAACACTTATCCGTAATGAGCCCGTTTTTGCCGCT
41       S  S  S  S  S  R  S  V  P  T  L  I  R  N  E  P  V  F  A  A

181     CCTGCTCCTATCATCACCCCTTACTGGAGCGAAGAGATGGGTAGCGAAGCATACGAAGAG
61       P  A  P  I  I  T  P  Y  W  S  E  E  M  G  S  E  A  Y  E  E

241     GCCATTGAAGCTCTCAAGAAGCTTATTATCGAGAAGGAGGAGCTAAAGACTGTTGCGGCC
81       A  I  E  A  L  K  K  L  I  I  E  K  E  E  L  K  T  V  A  A

301     GCTAAGGTGGAGCAGGCCACGGCAGCACTTCAGACAGGTACTTCCTCCGACAAGAAAGCT
101      A  K  V  E  Q  A  T  A  A  L  Q  T  G  T  S  S  D  K  K  A

361     TTCGACCCCGTCGAAAACATTAAGCAAGGCTTCATCACTTTCAAGAAGGAGAAATACGAA
121      F  D  P  V  E  N  I  K  Q  G  F  I  T  F  K  K  E  K  Y  E

421     ACCAACCCTGCTTTGTACGGTGAGCTCGCCAAGGGTCAAAGTCCTAAGTATATGGTGTTT
141      T  N  P  A  L  Y  G  E  L  A  K  G  Q  S  P  K  Y  M  V  F

481     GCTTGTTCCGACTCGCGTGTGTGTCCATCGCACGTTCTCAACTTTCAGCCAGGAGAGGCC
161      A  C  S  D  S  R  V  C  P  S  H  V  L  N  F  Q  P  G  E  A

541     TTCGTTGTCCGTAACATAGCCAACATGGTTCCTCCTTTTGACAAGGTCAAATACGGTGGA
181      F  V  V  R  N  I  A  N  M  V  P  P  F  D  K  V  K  Y  G  G

601     GTTGGAGCGGCCATCGAATACGCTGTTTTGCACCTTAAGGTGGAGAACATTGTGGTGATA
201      V  G  A  A  I  E  Y  A  V  L  H  L  K  V  E  N  I  V  V  I

661     GGACACAGCGCTTGTGGTGGGATCAAGGGACTTATGTCTTTTCCTTTGGATGGAAACAAC
221      G  H  S  A  C  G  G  I  K  G  L  M  S  F  P  L  D  G  N  N

721     TCCACTGATTTCATAGAGGACTGGGTCAAAATCTGTTTACCAGCCAAGTCAAAAGTCATA
241      S  T  D  F  I  E  D  W  V  K  I  C  L  P  A  K  S  K  V  I

781     TCAGAACTTGGAGATTCAGCCTTTGAGGACCAGTGTGGCCGATGTGAAAGGGAGGCAGTG
261      S  E  L  G  D  S  A  F  E  D  Q  C  G  R  C  E  R  E  A  V

841     AATGTTTCACTAGCAAACTTGTTGACATATCCATTCGTGAGAGAAGGACTTGTGAAAGGA
281      N  V  S  L  A  N  L  L  T  Y  P  F  V  R  E  G  L  V  K  G

901     ACCCTTGCTTTGAAGGGAGGTTACTATGATTTTATAAAGGGTGCTTTTGAGCTTTGGGGA
301      T  L  A  L  K  G  G  Y  Y  D  F  I  K  G  A  F  E  L  W  G

961     CTTGAGTTTGGCCTCTCCGAAACTAGCTCTGTATGA
321      L  E  F  G  L  S  E  T  S  S  V  *
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Fig. 1. The nucleotide sequence and the deduced amino acid sequence of β-CA from *Brassica napus* (GQ356780). Nucleotide and amino acid numbers are shown on the left-hand side.

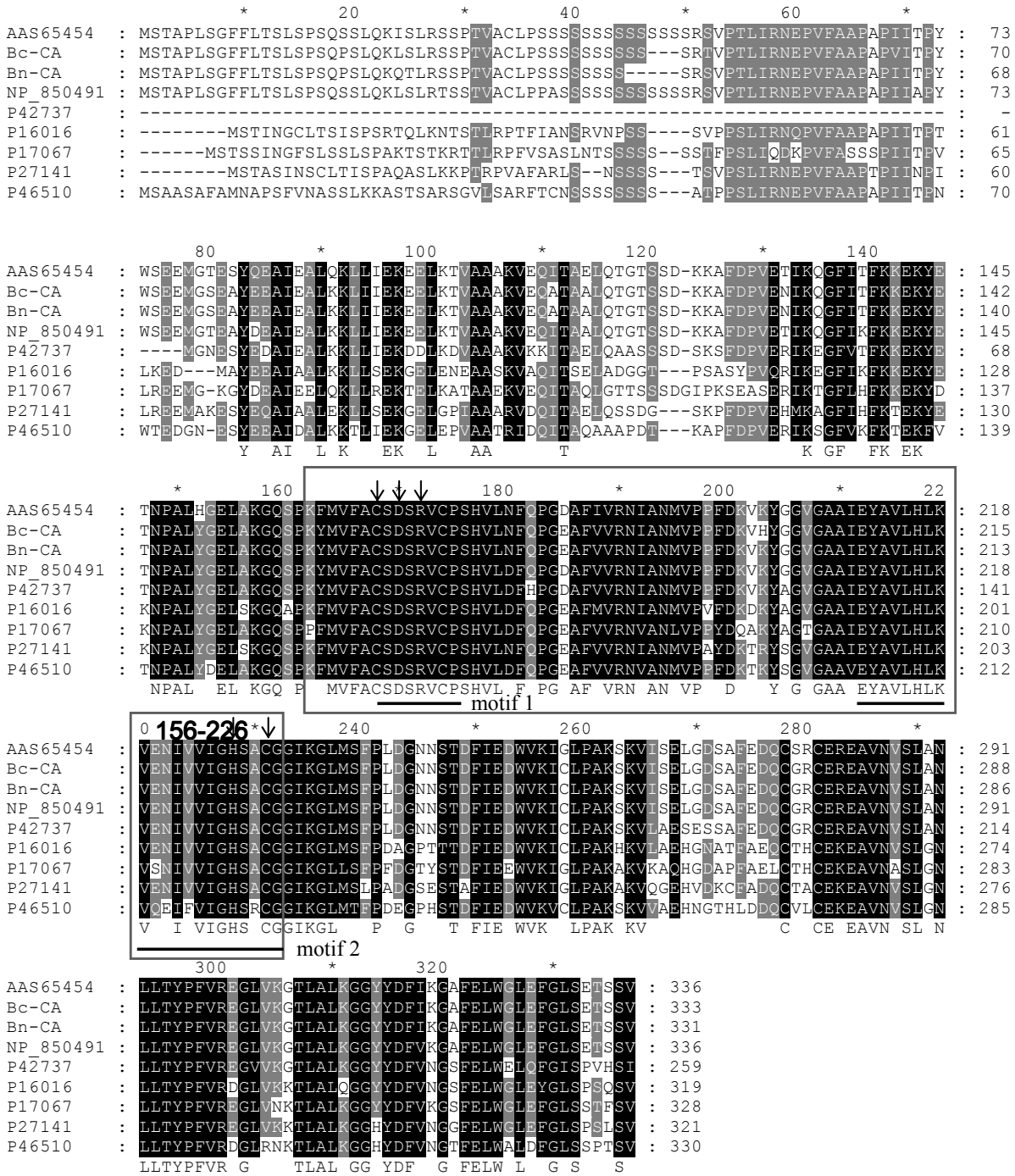

Fig. 2. Alignment of the putative amino acid sequence of β-CA from *Brassica napus* with those of other cloned β-CAs: AAS65454 (from *Thlaspi caerulescens*), Bc-CA (from *Brassica carinata*, being submitted to NCBI), Bn-CA (from *Brassica napus*, being submitted to NCBI), NP_850491 (CA1 from *Arabidopsis thaliana*), P42737 (CA2 from *Arabidopsis thaliana*), P16016 (from *Spinacia oleracea*), P17067 (from *Pisum sativum*), P27141 (from *Nicotiana tabacum*), P46510 (from *Flaveria bidentis*). Arrows point to five conserved residues (C, D, R, H, and C).

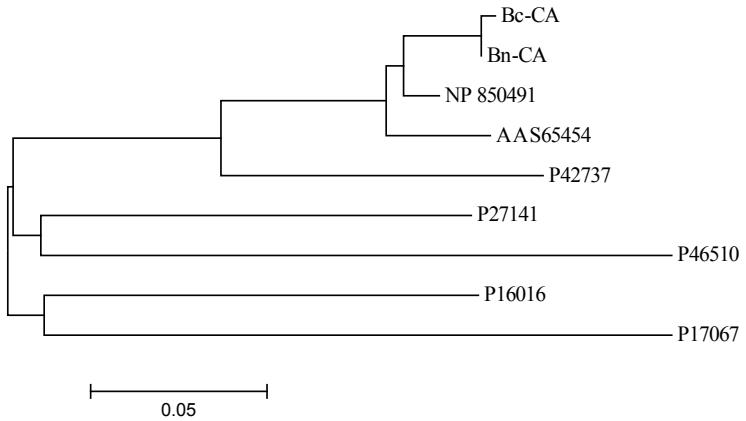

Fig. 3. Phylogenetic tree of sequence alignment of β-CAs from nine plants. The tree is built by the neighbour joining method according to Saitou and Nei (1987). The accession numbers of the β-CA sequences used are the same as in Fig. 2.

The successful expression of β-CA in prokaryotic cells lays a basis for further studies on function and structure of β-CA from *B. napus*. Further studies on functional expression and characterization of β-CA from *B. napus* will facilitate the detailed understanding of this putative β-CA gene.

Acknowledgements

This work was supported by grants from the National 863 High Technology Programs (grant number 2009AA101105), the New Century Excellent Talent Foundation of Ministry of Education in China (NCET-06-0646), and the National Basic Research Program of China (2006CB403301). We thank Associate Professor Lu Mingbo for providing the pET-32a vector and *E. coli* BL21 (DE3).

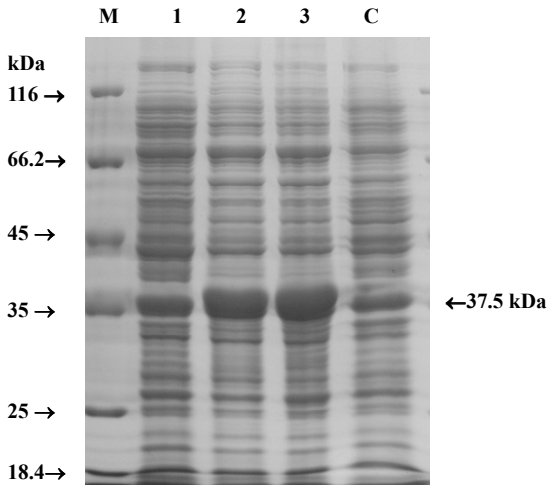

Fig. 4. 12% SDS-PAGE assay for β-CA expression induced by 1 mM IPTG. The total cell protein was analyzed on 12% SDS-PAGE stained with Coomassie brilliant blue. A 15-μl-protein sample was loaded. Lane M, low molecular weight standards; lanes 1, 2, 3, expression induction at 28 °C by addition of 1 mM IPTG for 0 h, 5 h, 10 h; lane C, expression induction at 28 °C by addition of 1 mM IPTG using the void vector pET-32a(+).

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