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# Highly active metallocarboxypeptidase from newly isolated *Geobacillus* strain SBS-4S: Cloning and characterization

Muhammad Tayyab,[1] Naeem Rashid,[1,*] Clement Angkawidjaja,[2] Shigenori Kanaya,[2] and Muhammad Akhtar[1,3]

*School of Biological Sciences, University of the Punjab, Quaid-e-Azam Campus, Lahore 54590, Pakistan,[1] Department of Material and Life Sciences, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita-shi, Osaka 565-0871, Japan,[2] and School of Biological Sciences, University of Southampton, Southampton, SO16 7PX, UK[3]*

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**The carboxypeptidase gene from *Geobacillus* SBS-4S was cloned and sequenced. The sequence analysis displayed the gene consists of an open reading frame of 1503 nucleotides encoding a protein of 500 amino acids ($CBP_{SBS}$). The amino acid sequence comparison revealed that $CBP_{SBS}$ exhibited a highest homology of 41.6% (identity) with carboxypeptidase Taq from *Thermus aquaticus* among the characterized proteases. $CBP_{SBS}$ contained an active site motif $^{265}$HEXXH$^{269}$ which is conserved in family-M32 of carboxypeptidases. The gene was expressed with His-Tag utilizing *Escherichia coli* expression system and purified to apparent homogeneity. The purified $CBP_{SBS}$ showed highest activity at pH 7.5 and 70°C. The enzyme activity was metal ion dependent. Among metal ions highest activity was found in the presence of $Co^{2+}$. Thermostability studies of $CBP_{SBS}$ by circular dichroism spectroscopy demonstrated the melting temperature of the protein around 77°C. The enzyme exhibited $K_m$ and $V_{max}$ values of 14 mM and 10526 μmol $min^{-1}$ $mg^{-1}$ when carbobenzoxy–alanine–arginine was used as substrate. $k_{cat}$ and $k_{cat}/K_m$ valves were 10175 $s^{-1}$ and 726 $mM^{-1}$ $s^{-1}$. To our knowledge this is the highest ever reported enzyme activity of a metallocarboxypeptidase and the first characterization of a metallocarboxypeptidase from genus *Geobacillus*.**



[**Key words:** Thermophiles; *Geobacillus* SBS-4S; Metallocarboxypeptidase; Circular dichroism; Melting point; Thermostability]

Carboxypeptidases are known to sequentially hydrolyze peptide bonds from the C-terminus of peptides/proteins. They are involved in protein degradation/turnover, processing of precursor proteins and metabolism of peptides/proteins in the biological systems. Outside the biological system, carboxypeptidases are being employed for C-terminal sequencing of proteins (1). Carboxypeptidases are classified on the basis of presence of active site residues into serine carboxypeptidases (E.C. 3.4.16), metallocarboxypeptidases (E.C. 3.4.17) and cysteine carboxypeptidases (E.C. 3.4.18). Metallocarboxypeptidases depend on divalent metal ions for their activity and have conserved either HEXXH or $HXXE(X)_{123-132}$ H active site motif (2).

Carboxypeptidases have been characterized from all the three domains of life including bacteria (3–5), eucarya (6) and archaea (7,8). However, no literature is available for the characterization of metallocarboxypeptidase from genus *Geobacillus*. In the present study we report characterization of a novel carboxypeptidase from a newly isolated *Geobacillus* strain SBS-4S. The microorganism was isolated from a hot spring located in the Northern Areas of Pakistan. This strain has the ability to produce a number of industrially important extracellular enzymes including amylase, lipase/esterase and protease (manuscript submitted). The carboxypeptidase being reported here is distinct from all other carboxypeptidases as it exhibits the highest ever reported enzyme activity. This is the first characterization of a metallocarboxypeptidase from genus *Geobacillus*.

## MATERIALS AND METHODS

**Reagents** All the chemicals were purchased from Sigma (Sigma-Aldrich Inc., St. Louis, MO, USA) and Wako (Wako Pure Chemical Industries, Osaka, Japan). Cbz–Ala–Arg was purchased from Bachem (Bachem AG, Bubendorf, Switzerland). DNA polymerase and restriction enzymes were purchased from Takara (Takara, Tokyo, Japan). The columns for purification were purchased from GE Healthcare (GE Healthcare Biociences, PA, USA). All other chemicals were commercial products of analytical or molecular biological grade.

**Cloning of carboxypeptidase** Carboxypeptidase gene was amplified by polymerase chain reaction (PCR) by using genomic DNA of SBS-4S as template and CBP-F (5'-CATATGAAACCAATCGAAGCGCAG) and CBP-R (5'-CTATAATCGATAAAGCGCC) as forward and reverse primers, respectively. These primers were designed on the basis of DNA sequence of carboxypeptidase from *Geobacillus kaustophilus* whose complete genome has been determined (9). The forward primer contained *Nde*I restriction site. The amplified PCR product was purified from the gel by DNA purification kit (Fermentas Life Sciences, Ontario, Canada), ligated in pTZ57R/T (Fermentas) using T4 DNA ligase (Fermentas). The resulting plasmid was named pTZ-cbp. *E. coli* DH5α cells were transformed using pTZ-cbp. Screening was done on the basis of blue/white color of the transformants (10). DNA sequencing of the cloned gene was performed using ABI-310 single capillary automated DNA sequencer (Applied Biosystems, Foster City, USA). The DNA and amino acid sequences of $CBP_{SBS}$ gene have been registered under accession numbers, AB457188 and BAH28805, respectively. Database homology searches were performed by using Basic Local Alignment Search Tool programme

* Corresponding author. Tel.: +92 42 99231534; fax: +92 42 99230980.
*E-mail addresses:* naeem.ff@sbs.pu.edu.pk, naeemrashid37@hotmail.com (N. Rashid).

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(11). Multiple sequence alignment and phylogenetic analyses were performed by using ClustalW programme (12) provided by DNA Data Bank of Japan.

**Expression of carboxypeptidase gene** In order to clone the gene in expression vector, recombinant pTZ-cbp was double digested with *Nde*I and *Hin*dIII. The restricted insert was purified from the agarose gel and ligated into pET-21a(+) (Novagen, Madison, WI, USA). The resulting plasmid was named pET21-cbp. *E. coli* DH5α cells were transformed using pET21-cbp. The transformants were screened by colony PCR and confirmed by digesting the purified recombinant plasmid pET21-cbp with restriction enzymes *Nde*I and *Hin*dIII. After confirmation, *E. coli* BL21 CodonPlus (DE3)-RIL (Novagen) cells were transformed using pET21-cbp. One of the colonies was inoculated in Luria Bertani medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) and grown overnight at 37°C. The overnight culture was diluted 100 times and incubated at 37°C. Expression of the carboxypeptidase gene was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when optical density of the culture at 660 nm reached 0.4 and incubation was carried out for another 4.5 h. The cells were collected by centrifugation at 8,000 rpm for 15 min and resuspended in 20 mM Tris–HCl buffer pH 8.0. The cells were lysed by using the French press (Thermo Electron Corporation, OH, USA). The protein samples were analyzed by denaturing polyacrylamide gel electrophoresis (SDS-PAGE). The expression of the gene was also examined at 25°C in the same way as described above except for the post induction time that was 20 h.

**Solubilization and refolding of carboxypeptidase** For refolding the recombinant carboxypeptidase, the inclusion bodies were solubilized in 6 M guanidinium hydrochloride (Gdn-HCl). Soluble and insoluble fractions were separated by centrifugation at 20,000 × *g* for 20 min. The soluble fraction after centrifugation was either diluted 100 times in the presence or absence of additives or dialyzed fractionally. Ionic (SDS) and nononic detergents (Tween-20, Tween-80, Triton X-100, Brij-35 and Brij-58) with a final concentration of 1% each, divalent cations (calcium, cobalt, magnesium, manganese and zinc) at a final concentration of 1 mM each, arginine 0.5 M, glycerol 2% and oxidized/reduced glutathione at 1 mM/0.2 mM concentrations, respectively, were used independently as additives in 20 mM Tris–HCl buffer pH 8.0 in order to refold the solubilized inclusion bodies.

**Production of carboxypeptidase with 6×-his-tag** The carboxypeptidase gene in pET21-cbp was digested with *Nde* I and *Hin*d III restriction enzymes and ligated in pET-28a previously digested with the same enzymes. The recombinant plasmid was named pET28-cbp. *E. coli* BL21 CodonPlus(DE3)-RIL cells were transformed using pET28-cbp. Heterologous expression of the gene was induced with 0.1 mM IPTG. Expression of the gene was analyzed by SDS-PAGE.

**Purification of carboxypeptidase** The soluble portion after cell lysis was loaded into the HiTrap Sepharose column (GE Healthcare) charged with $Ni^{2+}$ and equilibrated with 20 mM Tris–HCl pH 8.0 containing 150 mM NaCl and 5 mM imidazole. After loading the column was washed with the same buffer to remove all the unbound proteins. The bound protein was eluted by linearly increasing the concentration of imidazole to 500 mM. The purity of the protein was analyzed by SDS-PAGE. The fractions containing $CBP_{SBS}$ were mixed and concentrated by using amicon ultra (30,000 MWCO) filter assembly (Millipore, MA, USA). The concentrated protein was further purified by HiLoad 16/60 Superdex-200 (GE Healthcare) gel filtration column equilibrated with 20 mM Tris–HCl buffer pH 8.0.

**Enzyme activity assay** The activity of $CBP_{SBS}$ was determined by using Cbz–Ala–Arg as a substrate. The reaction mixture (250 μL) contained 2.5 μg $CBP_{SBS}$, 20 mM Tris–HCl buffer pH 8.0, 8 mM substrate and 1 mM cobalt chloride. This assay mixture was incubated at 70°C for 10 min followed by quenching on ice. The color was developed by the addition of 700 μL of $Cd^{2+}$-ninhydrin reagent with an incubation of 5 min at 80°C (7). Finally the samples were put on ice and optical density was measured at 500 nm. Enzyme activity was calculated by using L-arginine standard curve. The blank contained all the reagents except $CBP_{SBS}$. One unit of the activity was defined as the amount of enzyme that librates 1 μmol of L-arginine from Cbz–Ala–Arg per minute at 70°C.

**Effect of temperature on $CBP_{SBS}$** Effect of temperature on $CBP_{SBS}$ activity was examined at pH 8.0 in 20 mM Tris–HCl by incubating the assay mixture at various temperatures ranging from 40 to 80°C.

Thermostability and denaturation studies of $CBP_{SBS}$ were done by activity assay as well as circular dichroism (CD) spectroscopy. For thermostability analysis, the protein was incubated at 70°C in the presence and absence of various concentrations of $Co^{2+}$. Samples were withdrawn after every 15 min and the remaining enzyme activity was examined as described above.

For stability studies in the presence or absence of $Co^{2+}$, the CD value of the protein was analyzed at various temperatures ranging from 20 to 90°C at a wavelength of 222 nm. During these studies, an increase of 1°C/min was maintained. The far-UV CD spectrum of $CBP_{SBS}$ was analyzed at 20, 50, 70 and 90°C at a wavelength range of 200–260 nm. The protein used for thermostability/denaturation studies was in 20 mM Tris–HCl buffer pH 8.0 with a concentration of 0.7 mg/mL.

**Effect of pH, detergents and metal ions on the activity of $CBP_{SBS}$** For optimum pH, the enzyme activity was analyzed at 70°C and various pH in the presence of 100 mM of following buffers: sodium phosphate (pH 6–7) Tris–HCl (pH 7–9). In order to examine the pH stability of $CBP_{SBS}$, 10 μL of the protein sample was diluted 10 times in 100 mM of various buffers as mentioned above for optimal pH of the enzyme activity and incubated at 25°C for 1 h prior to the activity measurements. Activity assay was done by taking 10 μL of the above diluted sample and measuring the enzyme activity in 100 mM Tris–HCl buffer pH 8.0 at 70°C.

The effect of divalent metal ions on the enzyme activity was analyzed in the presence of 1 mM of each metal ions examined. Chloride salts of $Ca^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Fe^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Ni^{2+}$ and $Zn^{2+}$ were used.

In order to examine the effect of ionic and nononic detergents on $CBP_{SBS}$, activity assays were done individually in the presence 1% of each detergent.

**Molecular mass determination** Matrix-assisted laser desoption-ionization/time-of-flight mass spectrometry (MALDI-TOF MS) was used for molecular mass determination of recombinant $CBP_{SBS}$. Salts present in the protein sample were removed and 2.5 μL (2.5 μg) was mixed with 10 μL of 3, 5-dimethoxy-4-hydroxycinnamic acid (10 mg/mL in acetonitrile and 0.1% TFA water, in a ratio of 2:1). This mixture (1 μL) was applied to the plate and dried at room temperature for 15–25 min. Spectrum was observed using Voyager (ABI) in positive ion mode, by striking 200 shots in an acquisition mass range of 20,000–80,000 Da. Final spectrum was subjected to smoothing, baseline subtraction and centroiding.

## RESULTS

**Cloning of $CBP_{SBS}$** $CBP_{SBS}$ consisted of an open reading frame containing 500 amino acids. The amino acids sequence was utilized for homology comparison and phylogenetic tree construction (Fig. 1). $CBP_{SBS}$ shared 100% identity on the basis of amino acid sequence with uncharacterized carboxypeptidase from *G. kaustophilus*. Among the characterized carboxypeptidases, $CBP_{SBS}$ shared a highest homology of 41.6% with carboxypeptidase Taq from *Thermus aquaticus* YT-1 (6), 34.6% with carboxypeptidase PfuCP from *Pyrococcus furiosus* (13), 33.4% with carboxypeptidase 1 from *Thermococcus* NA1 (7), 12.8% with carboxypeptidase A from bovine pancreas (14), 12.4% with aminoacylase/carboxypeptidase from *Deinococcus radiodurans* (4), 12.2% with carboxypeptidase PP from *Pyrococcus horikoshii* (8), 11.4% with carboxypeptidase B from bovine pancreas (15), 11.2% with carboxypeptidase A1 from human (16), 10.4% with aminoacylase from *Geobacillus stearothermophilus* (17), 10% with carboxypeptidase A from *Sulfolobus solfataricus* (18) and 10% with carboxypeptidase Z from *Cyprinus carpio* (6). An amino acid sequence comparison of $CBP_{SBS}$ and its close homologues is shown in Fig. 2.

**Production of $CBP_{SBS}$ in *E. coli*** After induction by IPTG, *E. coli* BL21 CodonPlus(DE3)-RIL cells carrying pET21-cbp plasmid were lysed by sonication and the soluble and insoluble fractions were

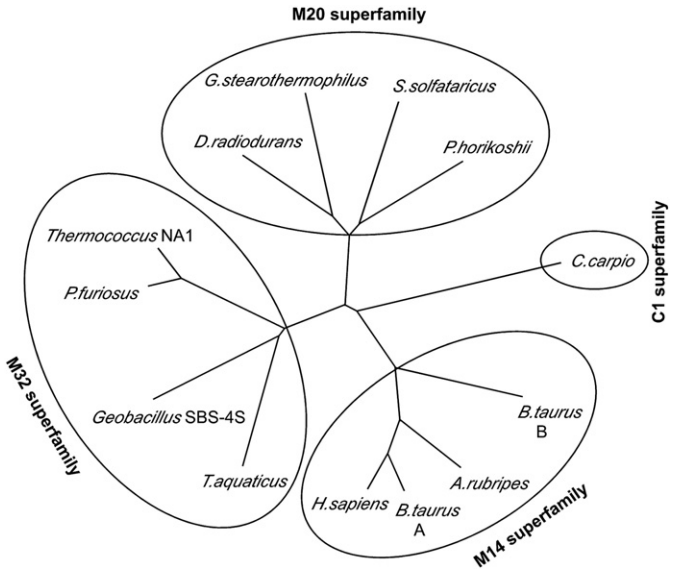


FIG. 1. Phylogenetic tree of $CBP_{SBS}$. The tree was constructed by using the already reported protein sequences available in various data bases. Following are the sequences with accession numbers used for the alignment in order to construct phylogenetic tree: SBS-4S from present study (BAH28805); *P. horikoshii* (AB009503); *Deinococcus radiodurans* R1 (AE001867); *Cyprinus carpio* (AY949988); *T. aquaticus* (P42663); *Thermococcus* sp. NA1 (DQ144135); *Sulfolobus solfataricus* (Z48497); *Homo sapiens* (AAH05279); *Takifugu rubripes* (NP_001163829); *Bos taurus* (P00730); *Bos taurus* (P00732); *G. stearothermophilus* (P37112) and *P. furiosus* (1K9X_C).

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P.furiosus      MEEVFQNETIKQILAKYRRIWAIGHAQS-VLGWDLEVNMPKEGILERSVAQGELSVLSHE  59
T.NA1           MEEVFQNETIKQILAKYRRIWAISHARS-VLGWDMEVNMPREGIFERSVAQGELSVLSQE  59
SBS-4S          ----MKPIEA-QFLQYVKKMTGYREAIG-LMYWDLRTGAPKKGVEQRSEVIGMLSEEVFR  54
T.aquaticus     ----MTPEAAYQNLLEFQRETAYLGSLGALAAWDQRTMIPRKGHGHRARQMAALARLLHE  56
                          * *                 **     *  *   *      *

P.furiosus      LLLHPEFVNLVEKAKG---LENLNEYERGIVRVLDRSIRIARAFPPEFIREVSETTSLAT 116
T.NA1           FLLKPEFVELVEKAKG---IEDLNEYERGVVRVLDRSIRISKSFPPEFLREMSEVTSQAT 116
SBS-4S          MSTSEEMAAFIAKLSPKAVYEQLNDVTKKTLDECKKEYERNKKIPADEYKEFVVLCSKAE 114
T.aquaticus     RATDPRIGEWLEKVEGSSLVEDPLSDAAVNVRAWRRAYERARAIPERLAVELAQARSEGE 116
                          *       *                       *    *    *

P.furiosus      KAWEEAKAKDDFSKFEPWLDKIISLAKRAAEYLGYEE---------EPYDALLDLYEEGL 167
T.NA1           KAWEEAKRTNDYSKFEPWLDRIIDLAKRAADYLGYED---------EPYDALLDLFEEGT 167
SBS-4S          SVWEEAKAAADFARFRPYLEQIIEFQRRFIRYWGYEG---------HPYNTLLDQYEPGM 165
T.aquaticus     TAWEALRPRDDWQGFLPYLKRLFALAKEEAEILMAVGPDPLDPPYGELYDALLDGYEPGA 176
                  **      *   * * *                           *  ***  * *

P.furiosus      RTRDVEKMFEVLEKKLKPLLDKILEEGKVPREHPLEKEKYEREWMERVNLWILQKFGFPL 227
T.NA1           TTRDVERMFKKLEKELKPLLEKIMDEGKVPQSHPLEKEKYKREQMERVNLWILEKFGFPL 227
SBS-4S          TVDLLDELFSRLRERIVPLVHAISAASDKPDTSFLFAP-FPKEKQRAFLLELLKELGYDF 224
T.aquaticus     RARDLEPLFRELSSGLKGLLDRILGSGRRPDVGVLHRH-YPKEAQRAFALELLQACGYDL 235
                        *  *      *   *      *    *       *      *  *   *

P.furiosus      GTRARLDVSA**HPF**TTEFGIR**DVRIT**TRYEGYDFRRTILSTV**HE**FG**H**ALYELQQDERFMFT 287
T.NA1           GVRSRLDVSA**HPF**TTEFGIR**DVRIT**TRYEGYDFRRTILSTV**HE**FG**H**ALYELQQDERFMFS 287
SBS-4S          GK-GRLDETV**HPF**AIGLNPN**DVRIT**TRYDERDFRTAVFGTI**HE**CG**H**ALYEQHISEALVGT 283
T.aquaticus     EA-GRLDPTA**HPF**EIAIGPG**DVRIT**TRYYEDFFNAGIFGTL**HE**MG**H**ALYEQGLPEAHWGT 294
                    ***   ***       ********    *      * ** ******    *

P.furiosus      PIAGGVSLGI**HESQ**SRFWENIIGRSKEFVELIYPVLKENLPF-MSNYTPEDVYLYFNIVR 346
T.NA1           PIAGGVSLGI**HESQ**SRFWENVIGRSREFAELIHPVLKENLPF-MANYTPEDVYLYFNMVR 346
SBS-4S          PLASGASMGI**HESQ**SLFFENMIGRHYAFWKRHYPRLQQYAPTQFADVSLDAFYRAINEAK 343
T.aquaticus     PRGEAASLGV**HESQ**SRTWENLVGRSLGFWERFFPRAKEVFSS-LADVRLEDFHFAVNAVE 353
                *     * * *****   **  **   *     *                       *

P.furiosus      PDF**IR**T**EAD**VVTYNFHILLRFKLERLMVSEEIKAKDLPEMWNDEMERLLGIRPRKYSE   404
T.NA1           PDF**IR**T**ESD**VVTYNFHILLRFKLERMMLNEGVKAKDLPELWNEEMERLLGIRPKTYAE   404
SBS-4S          PSL**IR**I**EAD**ELTYPLHIIIRYEIEKQLFAGELEAIDLPDVWNEKYEQYLGIRPHNDAV   401
T.aquaticus     PSL**IR**V**EAD**EVTYNLHILVRLELELALFRGELFLEDLPEAWREKYRAYLGVAPRDYKD   411
                *  ** * *  **  **  *   *           ***  *       **  *

P.furiosus      **GILQDIHW**AHGSIGYFPTYTIGTLLSAQLYYHIKKDIPDFEEKVAKAEFDPIKAWLREKI 464
T.NA1           **GILQDIHW**AHGTVGYFPTYSIGTLLSAQIYYHMKRDIPDFEEKVARAEFEPIKAWLREKI 464
SBS-4S          **GVLQDVHW**SGGSFGYFPSYALGYMYAAQFKQAMEKELD-VAGLLEEGNIAPIREWLTVHI 460
T.aquaticus     **GVMQDVHW**SGGMFGYFPTYTLGNLYAAQFFAKAQEELGPLEPLFARGEFTPFLDWTRRKI 471
                *  ** **  *  **** *  *    **                     *   *    *

P.furiosus      HRWGSIYPPKELLKKAIGEDMDAEYFVRWVKEKYL----- 499
T.NA1           HRWGSIYPPKDLLKKAIGEELNPEYFVRWVKERYL----- 499
SBS-4S          HQFGKMKKPLELVRDATGETLKADYLIQYLEEKYKALYRL 500
T.aquaticus     HAEGSRFRPRALVERVTGSPPGAQAFLRYLEAKYGALYGF 511
                *  *    *  *     *               *
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FIG. 2. Amino acid sequence comparison of $CBP_{SBS}$ with its closest homologues. Identical amino acids are shown by asterisks below the sequence. The names at the left hand side indicate the organism from which the sequence originated. The conserved domains are shown by bold letters. The active site motif belonging to family-M32 of carboxypeptidases is under lined. Gaps are shown by dashes. The accession numbers are: SBS-4S from present study (BAH28805); *T. aquaticus* (P42663); *Thermococcus* sp. NA1 (DQ144135) and *P. furiosus* (1K9X_C).

analyzed by SDS-PAGE. The analysis showed that almost 90–95% of recombinant $CBP_{SBS}$ was produced as inclusion bodies. Attempts to get the recombinant $CBP_{SBS}$ in soluble form by growing and inducing the host cells at lower temperature (17–23°C) were unsuccessful. After failures in producing recombinant $CBP_{SBS}$ in the soluble and enzymatically active form, we tried to solubilize the inclusion bodies by completely denaturing the recombinant protein and refolding it in the absence or presence of various additives as given in the materials and methods section but in vain.

We mentioned above that about 5–10% of the recombinant $CBP_{SBS}$ was produced in *E. coli* in the soluble form. In order to facilitate the purification of enzymatically active protein from the soluble fraction of *E. coli* cells, we added a His-Tag at the N-terminal of the protein. When His-$CBP_{SBS}$ was produced in *E. coli* at 25°C, a 3-fold higher amount was produced in soluble and active form compared to 37°C (data not shown). His-$CBP_{SBS}$ from the soluble fraction was purified by $Ni^{2+}$-affinity column chromatography and gel filtration. SDS-PAGE analysis demonstrated that His-$CBP_{SBS}$ had a molecular weight of

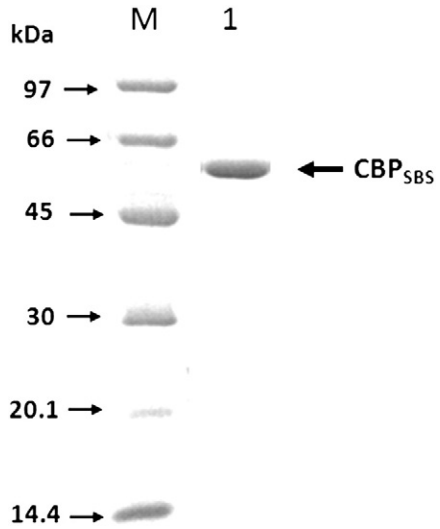


FIG. 3. Coomassie brilliant blue stained 15% SDS-PAGE showing the purified recombinant CBP$_{SBS}$. Lane M, molecular weight marker; lane 1, purified protein. Approximately 5 μg protein was loaded in lane 1.

58 kDa (Fig. 3). Molecular weight of recombinant His-CBP$_{SBS}$ was found to be 58,464 Da when determined by MALDI-TOF spectrometery (Fig. 4) which agreed well with the molecular weight calculated based on the amino acid sequence of the protein. When passed through gel filtration column His-CBP$_{SBS}$ eluted at a retention volume of 70 mL corresponding to a molecular weight of 120 kDa indicating that His-CBP$_{SBS}$ exists in a dimeric form in solution.

**Enzyme characterization** When we performed the enzyme activity assay at various temperatures at pH 8.0, the activity of His-CBP$_{SBS}$ increased with the increase in temperature and the highest activity was found at 70°C (Fig. 5A). Optimal pH for the CBP$_{SBS}$ activity was found to be 7.5 (Fig. 5B) in Tris–HCl. Regarding the pH stability, it was found that His-CBP$_{SBS}$ was quite stable at a wide pH range (data not shown).

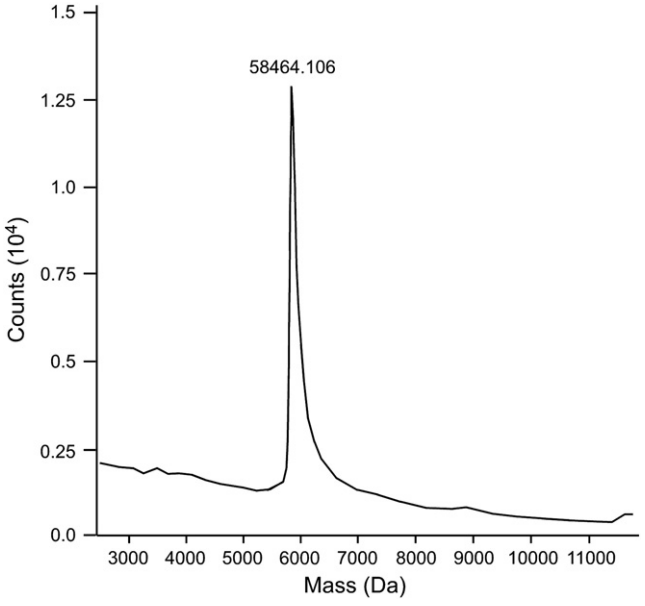


FIG. 4. MALDI-TOF mass spectrum of the purified recombinant CBP$_{SBS}$. The counts are shown on the *Y*-axis, and the deconvoluted mass in 10 X daltons on *X*-axis. Molecular mass of the recombinant CBP$_{SBS}$ is written at the top of the peak.

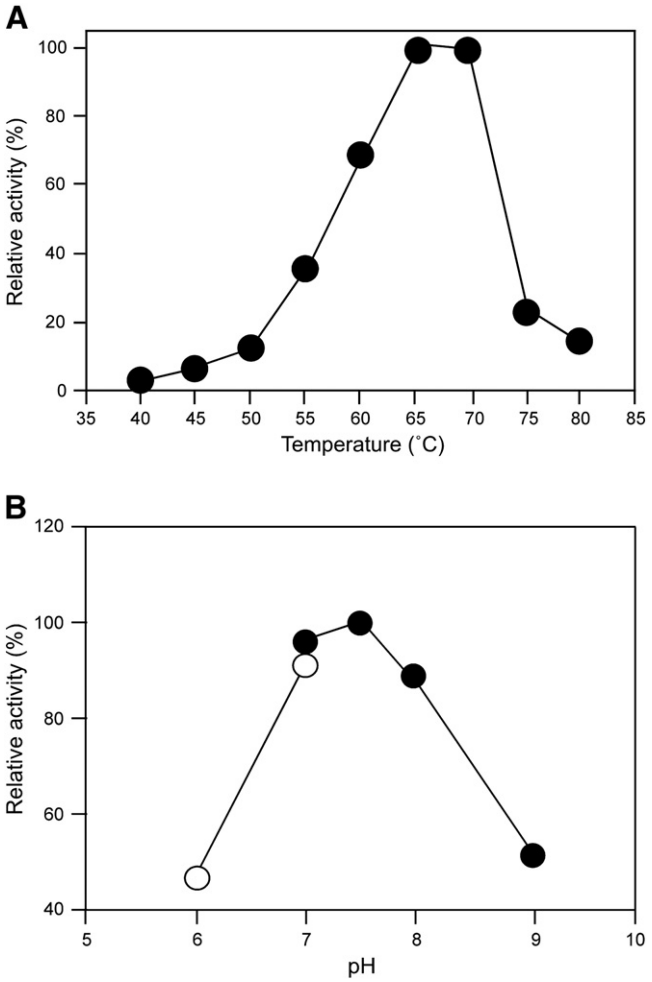


FIG. 5. Effect of temperature and pH on CBP$_{SBS}$ activity. (A) Optimal temperature for enzyme activity. The activity assays were done at various temperatures (40– 80°C) by using Cbz–Ala–Arg as substrate in 20 mM Tris–HCl buffer at pH 8.0 containing 1 mM $Co^{2+}$. (B) Effect of pH on activity of CBP$_{SBS}$. Activity assays were performed in 100 mM phosphate buffer (open circles) or Tris–HCl buffer (closed circles) at 70°C.

We also examined the effect of divalent metal cations on the activity of His-CBP$_{SBS}$. The presence of $Cu^{2+}$, $Fe^{2+}$, $Ni^{2+}$ or $Zn^{2+}$ at a final concentration of 1 mM showed an inhibitory effect. The enzyme activity was remarkably enhanced in the presence of $Co^{2+}$ (12-fold)

**TABLE 1.** Effect of various metal ions and detergents on the enzyme activity.

| Divalent cation or EDTA or detergent | Relative activity (%) |
|---|---|
| None | 100 |
| EDTA (1 mM) | 0 |
| Metal[a](1 mM) | |
| $Zn^{2+}$ | 9 |
| $Cu^{2+}$ | 9 |
| $Fe^{2+}$ | 12 |
| $Ni^{2+}$ | 13 |
| $Mg^{2+}$ | 115 |
| $Ca^{2+}$ | 119 |
| $Mn^{2+}$ | 668 |
| $Co^{2+}$ | 1200 |
| Detergent (1%) | |
| Triton X-100 | 278 |
| Tween 20 | 111 |
| Tween 80 | 110 |
| Brij-35 | 26 |
| SDS | 3 |

[a]Metal chlorides were used in the assay.

**TABLE 2.** Comparison of kinetic parameters of various carboxypeptidases.

| Origin | Substrate | Specific activity (U $mg^{-1}$) | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | Temperature (°C) |
|---|---|---|---|---|---|
| *Geobacillus* SBS-4S | Cbz–Ala–Arg | 9383 | 14 | 10175 | 70 |
| *Thermus aquaticus* | Cbz–Phe–Tyr | 3000 | NA | NA | 70 |
| *Pyrococcus furiosus* | Cbz–Ala–Arg | 1,391 | 0.9 | 600 | 80 |
| *Thermococcus* sp. *NA1* | Cbz–Ala–Arg | NA | 1.6 | 12.8 | 70 |
| *Deinococcus radiodurans* | Cbz–Gly–Ala | 15.15 | 4.3 | 28 | 40 |
| *Pyrococcus horikoshii* | Cbz–Gly–Phe | 119 | 7.35 | 91.4 | 85 |

The substrates used were carbobenzoxy–alanine–arginine (Cbz–Ala–Arg), carbobenzoxy–phenyl–tyrosine (Cbz–Phe–Tyr), carbobenzoxy–glycine–alanine (Cbz–Gly–Ala) and carbobenzoxy–glycine–phenylalanine (Cbz–Gly–Phe). The parameters not available in literature are shown by NA. The data are from the present study or *T. aquaticus* (6), *P. furiosus* (12), *Thermococcus* sp. *NA1* (7), *D. radiodurans* (4) and *P. horikoshii* (8).

and $Mn^{2+}$ (7-fold) at a final concentration of 1 mM (Table 1). No activity could be detected when enzyme assay was performed in the presence of 1 mM EDTA indicating that the enzyme activity is dependent on divalent metal cation(s). As the enzyme activity remarkably enhanced in the presence of 1 mM $Co^{2+}$ we therefore examined the effect of various concentrations of $Co^{2+}$ on the enzyme activity and found highest activity in the presence of 100 μM $Co^{2+}$.

The effect of ionic and nononic detergents on His-$CBP_{SBS}$ enzyme activity was also examined. The enzyme activity was totally abolished in the presence of 1% SDS. Among the nononic detergents, presence of 1% of Tween-20, Tween-80 and Brij-58 did not show any significant effect. Presence of 1% Brij-35 in the assay mixture showed an inhibitory effect. A 3-fold enhancement in the enzyme activity was observed in the presence of 1% Triton X-100 (Table 1). Enzyme activity of several carboxypeptidases, particularly membrane bound, has also been reported to be enhanced with the addition of triton X-100 (19,20). $CBP_{SBS}$ exhibited a specific activity of 9383 U $mg^{-1}$ (Table 2) when assay was conducted in 20 mM Tris–HCl at 70°C and pH 7.5 in the presence of 100 μM $Co^{2+}$.

**Kinetic parameters** For examining the kinetic parameters various concentrations of carbobenzoxy–alanine–arginine ranging from 1 to 60 mM were used and assay was conducted under optimal conditions. The enzyme followed the Michaelis-Menten equation (Fig. 6). $K_m$ and $V_{max}$ values were found to be 14 mM and 10526 μmol $min^{-1}$ $mg^{-1}$, respectively. $k_{cat}$ was calculated on the basis of monomeric form of His-$CBP_{SBS}$ and was found to be 10175 $s^{-1}$.

**Thermostability analysis of His-$CBP_{SBS}$** Thermostability of His-$CBP_{SBS}$ was analyzed at 70°C in the absence or presence of various concentrations of $Co^{2+}$ (data not shown). There was no significant difference of thermostability with or without the addition of 100 μM $Co^{2+}$. Half-life of His-$CBP_{SBS}$ activity without the addition of any metal ion was 110 min at 70°C. In the presence of >100 μM $Co^{2+}$, the thermostability of His-$CBP_{SBS}$ decreased gradually with the increase in $Co^{2+}$ concentration. In the presence of 500 μM $Co^{2+}$, the residual activity was only 5% after an incubation of 120 min at 70°C.

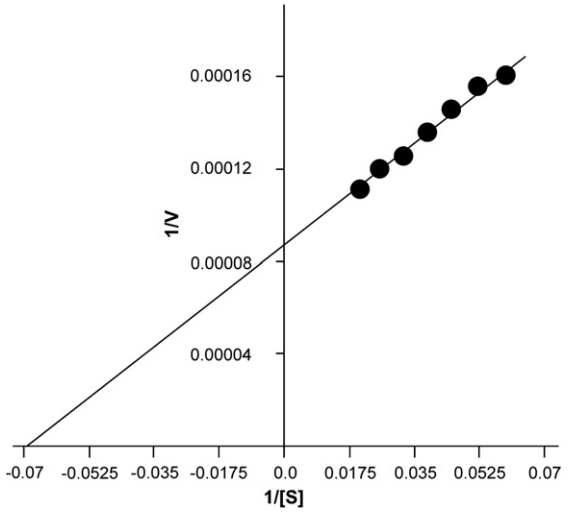


FIG. 6. Lineweaver–Burk plot obtained by taking the inverse of the substrate concentrations (mM) along *X*-axis and velocities (μmol $min^{-1}$ $mg^{-1}$) along *Y*-axis.

The melting temperature and thermostability of His-$CBP_{SBS}$ were also analyzed in the absence and presence of 100–500 μM $Co^{2+}$ by CD spectroscopy. The melting temperature in the absence of $Co^{2+}$ was found to be 76.9°C. There was no significant difference in the melting temperature in the presence of $Co^{2+}$ (Fig. 7A). When we examined the CD spectrum of the protein by gradually lowering the incubation temperature from 90 to 20°C we found that heat-induced denaturation was irreversible. We further analyzed the far-UV spectra of His-$CBP_{SBS}$ at various temperatures ranging from 20 to 90°C. The molecular ellipticity gradually shifted towards zero with the increase in temperature indicating either conformational or secondary structural changes at high temperature rendering the protein inactive (Fig. 7B).

## DISCUSSION

Carboxypeptidases from several mesophilic and thermophilic strains have been characterized. The closet homologue of $CBP_{SBS}$, carboxypeptidase Taq from *T. aquaticus*, belongs to peptidase family-M32. Several conserved domains including HEXXH, DXRXT, HPF, HESQ, IRXXAD and GXXQDXHW, proposed to be involved in metal ion and substrate binding or activity of thermostable carboxypeptidases (7) were also found in the $CBP_{SBS}$ sequence (Fig. 2). The sequence alignment demonstrated that in $CBP_{SBS}$ $^{265}$HECGH$^{269}$ constituted the conserved HEXXH domain found in family-M32 carboxypeptidases. The conserved motif HXXE$(X)_{123-132}$H present in M14-like superfamily of carboxypeptidases was also found in $CBP_{SBS}$ ($^{275}$HXXE$(X)_{129}$H$^{408}$). At present we don't know whether this sequence is really a conserved motif found in M14-like superfamily or it is formed accidentally. In order to know if this motif is important for enzyme activity mutational studies in this region particularly at $H^{275}$ are required.

Kinetic parameters of His-$CBP_{SBS}$ are compared with other characterized carboxypeptidases in Table 2. Among the closest homologues of His-$CBP_{SBS}$ highest enzyme activity (3000 U/mg) has been reported for carboxypeptidase Taq when Cbz–Phe–Tyr was used as a substrate (2). Carboxypeptidase from *P. furiosus*, the second closest homologue of $CBP_{SBS}$, displayed a $V_{max}$ value of 2300 U/mg using the same substrate (Cbz–Ala–Arg) as was used in case of $CBP_{SBS}$ (13). Although $CBP_{SBS}$ exhibited a much higher value of $V_{max}$ compared to *P. furiosus* carboxypeptidase, catalytic efficiency ($k_{cat}/K_m$) of both the enzymes was similar (around 700 $mM^{-1}$ $s^{-1}$). The third closest homologue of $CBP_{SBS}$ was carboxypeptidase from *Thermococcus* sp. NA1 and it had 87-fold lower catalytic efficiency (8.3 $mM^{-1}$ $s^{-1}$) compared to $CBP_{SBS}$. The higher enzyme activity of $CBP_{SBS}$ might be correlated with the presence of two active site motifs. Further work on mutational studies in

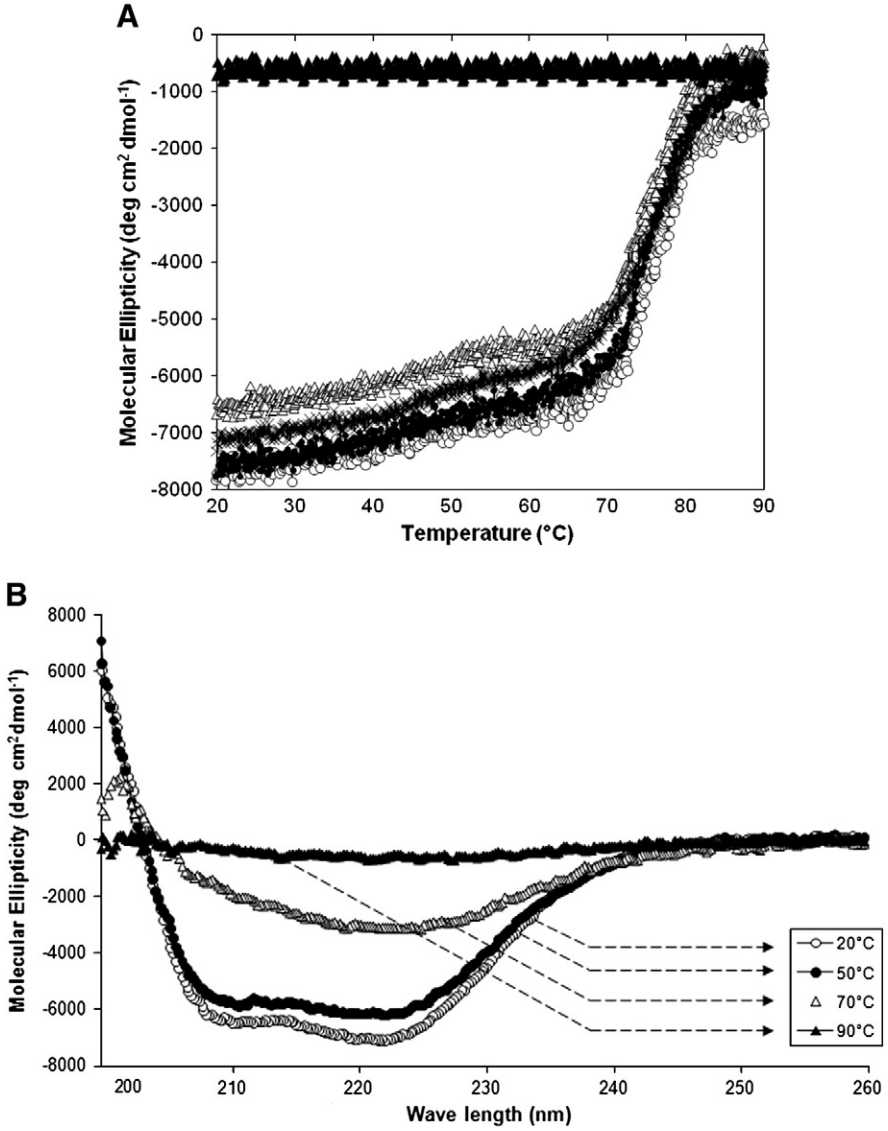


FIG. 7. Circular dichroism studies on $CBP_{SBS}$. (A) Thermostability and melting temperature of $CBP_{SBS}$. Thermostability was analyzed from 20 to 90°C at a wavelength of 222 nm in the absence (crosses) or presence of 100 μM (open circles), 200 μM (closed circles) and 500 μM (open triangles) $Co^{2+}$. The reversibility of denaturation (closed triangles) was analyzed by decreasing the temperature from 90 to 20°C. (B) Far-UV spectrum of $CBP_{SBS}$. The denaturation of $CBP_{SBS}$ was analyzed by examining the absorption spectra from 200 to 260 nm at 20°C (open circles), 50°C (closed circles), 70°C (open triangles) and 90°C (closed triangles).

the active site domains and crystallographic analysis will shed light whether both the domains are responsible for the observed activity or not.

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