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REVIEW

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Alkaline detergent enzymes from alkaliphiles: enzymatic properties, genetics, and structures

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Abstract The cleaning power of detergents seems to have peaked; all detergents contain similar ingredients and are based on similar detergency mechanisms. To improve detergency, modern types of heavy-duty powder detegents and automatic dishwasher detergents usually contain one or more enzymes, such as protease, amylase, cellulase, and lipase. Alkaliphilic *Bacillus* strains are often good sources of alkaline extracellular enzymes, the properties of which fulfil the essential requirements for enzymes to be used in detergents. We have isolated numbers of alkaliphilic *Bacillus* that produce such alkaline detergent enzymes, including cellulase (CMCase), protease, α-amylase, and debranching enzymes, and have succeeded in large-scale industrial production of some of these enzymes. Here, we describe the enzymatic properties, genetics, and structures of the detergent enzymes that we have developed.

Key words Alkaliphile · *Bacillus* · Detergent enzyme · α-Amylase · Debranching enzyme · Protease · Cellulase

Introduction

Alkaliphilic *Bacillus* strains often produce various alkaline enzymes, including alkaline CMCase, protease, and α-amylase (Horikoshi and Akiba 1982; Horikoshi 1996). Various alkaline proteases have long been incorporated as biobuilders into heavy-duty detergents to hydrolyze and remove proteinaceous materials in soiled clothes (Horikoshi 1971; Hoshino et al. 1995; Kobayashi et al. 1995). To clean starch-base stains, amylases are included in some heavy-duty detergents and automatic dishwashing detergents, especially in Europe. Alkaline CMCase from an alkaliphilic *Bacillus* was included in Japanese detergents for the first time by us (Ito 1997). In this review, we describe alkaline enzymes for use in detergents we have developed, with emphasis on their enzymatic properties, genetics, and structures.

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High-alkaline proteases from alkaliphilic *Bacillus* sp. KSM-K16

Properties of multiple alkaline proteases produced by the organism

Alkaliphilic *Bacillus* sp. KSM-K16 produces three alkaline serine proteases, designated M-, H-, and N-proteases, as determined by polyacrylamide gel electrophoresis (PAGE) (Kobayashi et al. 1996). Such multiple electrophoretic forms of alkaline proteases are also observed for other commercially available laundry proteases known as Savinase, Esperase, and Maxacal and in the case of no. 221 protease from *B. alcalophilus* ATCC21522 (Horikoshi 1971). Zuidweg et al. (1972) also detected three protease components in a detergent enzyme, Maxatase, during isoelectrofocusing PAGE of the enzyme.

The major M-protease was purified to homogeneity and characterized (Kobayashi et al. 1995). The molecular mass and isoelectric point of M-protease were approximately 28 kDa and higher than pH 10.6, respectively. The specific activity toward casein was 127 units (mg protein)$^{-1}$. Maximum activity was observed at 55°C and at pH 12.3 in phosphate-NaOH buffer. These properties of M-protease are essentially comparable to those of AH-101 (Takami et al. 1990), no. 221, and PB92 (Nijenhuis 1977) proteases. Characteristically, M-protease acted on naturally occurring proteins, such as keratin and elastin, and had cleavage specificity for the oxidized insulin B chain different from and broader than those of any other alkaline serine proteases, such as subtilisins Carlsberg (Johansen et al. 1968) and BPN′ (Morihara and Tsuzuki 1969), no. 221 (Horikoshi and Akiba 1982), ALPase I (Tsuchida et al. 1986), BYA

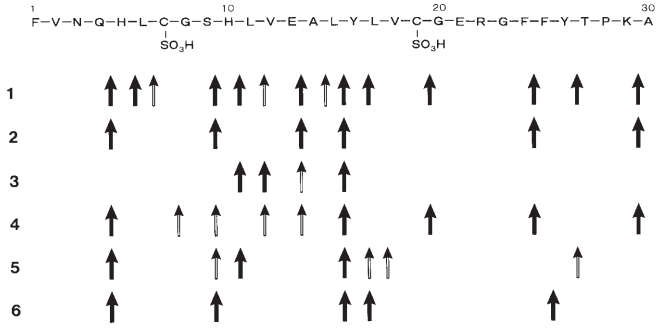

Fig. 1. Pattern of cleavage by M-protease of the oxidized insulin B chain and a comparison with those of other alkaline proteases. *1*, M-protease (Kobayashi et al. 1995); *2*, no. 221 (Horikoshi and Akiba 1982); *3*, AH-101 (Takami et al. 1992); *4*, alkaline elastase YaB (Tsai et al. 1988); *5*, subtilisin Carlsberg (Johansen et al. 1968); *6*, subtilisin BPN′ (Morihara and Tsuzuki 1969). *Closed arrows*, major cleavage sites; *open arrows*, minor sites

(Shimogari et al. 1991) and AH-101 (Takami et al. 1992) proteases, and alkaline elastase YaB (Tsai et al. 1988) (Fig. 1). The enzyme cleaved the insulin initially at Leu15–Tyr16, and the other principal cleavage sites were the carbonyl bonds of Gln4, His5, Ser9, His10, Glu13, Tyr16, Cys19, Phe24, Tyr26, and Lys29. The enzyme also acted on the carbonyl bonds of Leu6, Leu11, and Ala14, although to a lesser extent. It could hydrolyze various synthetic oligopeptidyl *p*-nitro-anilides (pNA) and had at least five substrate-binding sites (S1–S5). The good substrates, such as Suc-Ala-Ala-Pro-Phe-pNA, Suc-Ala-Ala-Pro-Met-pNA, and Suc-Ala-Ala-Val-Ala (where Suc represents *N*-succinyl), with catalytic efficiencies (k_{cat}/K_m) of 174.4, 137.3, and 129.3 s^{-1} mM^{-1}, respectively, may interact with the putative S2–S4 subsites. The number of subsites in the M-protease molecule is identical to those in reported alkaline serine proteases, such as ALPase II (Yamagata and Ichishima 1989) and AH-101 (Takami et al. 1992) proteases.

To analyze the multiple electrophoretic forms of alkaline protease from *Bacillus* sp. KSM-K16, we also purified two minor H- and N-proteases to homogeneity (Kobayashi et al. 1996). The molecular mass of H-protease was 28 kDa. Catalytic properties of H-protease (specific activity 120) were essentially similar to those of M-protease. N-Protease, having a very low specific activity (3.8), was composed of two polypeptides with molecular masses of 12.5 and 14.5 kDa, as judged by sodium dodecyl sulfate- (SDS-) PAGE, although it migrated as a single band of protein during nondenaturing PAGE. N-Protease appears to be the autolytic product of M- or H-proteases.

Possible interrelationship between M- and H-proteases

The N-terminal amino acid sequences of H-protease and of the 14.5-kDa polypeptide of N-protease were identical to that of M-protease. Polyclonal antibodies raised against the purified M- and N-proteases reacted with all three enzymes in Western blotting analysis. Electrophoretic analysis showed that, even in the presence of phenylmethylsulfonyl fluoride, M-protease was gradually converted to H- and N-proteases, and H-protease was converted to M-protease much more rapidly than to N-protease, when each was stored at pH 8.0. Considering the α-helix contents of M-protease (21.3%; Kobayashi et al. 1995) and H-protease (26.3%; Kobayashi et al. 1996) and the rapid conversion of H-protease to M-protease at pH 8.0, we postulated that H-protease could not be converted to M-protease at high pH values, namely pH values higher than pK_a values of α-helical amino acid residues. In fact, H-protease was not converted to M-protease at pH 11, while it was converted to M- and N-proteases at pH 5.0. By contrast, M-protease was converted to H- and N-proteases more rapidly at pH 11. The conformational difference between M- and H-proteases could represent the destabilization or stabilization of an α-helical structure by some amino acid residues, because the conversion from H-protease to M-protease was not observed at pH 11. As no Cys residues (its thiol group has a high pK_a value) are found in the deduced amino acid sequence (Hakamada et al. 1994) and in the amino acid composition of M-protease (Kobayashi et al. 1995), Lys or Tyr residues, the pKa values being slightly higher than pH 11, may be related to the conformational difference between the two forms of our enzyme at different pH values. When the recombinant M-protease was expressed in *B. subtilis* cells, three active proteases, corresponding to M-, H-, and N-proteases, could also be detected in the cultures (Kobayashi et al. 1996). Multiple forms of Savinase, Esperase, Maxacal, and no. 221 protease may be generated through similar mechanism(s) with our enzyme. The multiple form, reversibly convertible by change in pH, has also been found in two acid phosphatase components of rice grains (Yamagata et al. 1979).

Crystal structure and high-alkaline adaptation mechanism

The gene for M-protease was cloned and sequenced, and the nucleotide sequence determined included an open reading frame (ORF) of 1143 bp (380 amino acids) that encoded a pre-pro-peptide (111 amino acids) and a mature protein (269 amino acids; 26 723 Da) (Hakamada et al. 1994). The catalytic triad and the subsite sequence in subtilisin BPN′ were well conserved as Asp32, His62, and Ser215 and as Ser123-Leu124-Gly125, respectively, in M-protease (Fig. 2). However, M-protease exhibited relatively low homology to subtilisins BPN′ (58.7%; Wells et al. 1983) and Carlsberg (59.5%; Jacobs et al. 1985).

The three-dimensional structure of M-protease has been determined (Yamane et al. 1995; Shirai et al. 1997a) and is essentially similar to other subtilisins of known structure where eight helices form a basic skeleton. In the high-resolution electron density map shown in Fig. 3a, the catalytic triad of M-protease is composed of Asp32, His62, and Ser215, and a hydrogen bond is not found between His,$N^{\varepsilon 2}$ and Ser,O^{γ} of the catalytic triad. In the crystal, the molecule has a salt bridge between Arg19, Arg269, and Glu265 (Fig. 3c), which is located on the opposite side from the catalytic site. These two Arg residues are found only in high-alkaline

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                                                30
AQSVPWGISRVQAPAAHNRGLTGSGVKVAV
                                                60
LDTGISTHPDLNIRGGASFVPGEPSTQDGN
                                                90
GHGTHVAGTIAALNNSIGVLGVAPSAELYA
                                               120
VKVLGASGSGSVSSIAQGLEWAGNNGMHVA
                                               150
NLSLGSPSPSATLEQAVNSATSRGVLVVAA
                                               180
SGNSGAGSISYPARYANAMAVGATDQNNNR
                                               210
ASFSQYGAGLDIVAPGVNVQSTYPGSTYAS
                                               240
LNGTSMATPHVAGVAALVKQKNPSWSNVQI
                                               269
RNHLKNTATGLGNTNLYGSGLVNAEAATR
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Fig. 2. Deduced amino acid sequence of the mature M-protease. The amino acid residues of the catalytic triad (Asp32, His62, and Ser215) are *boxed*. The putative subsite sequence (Ser123-Leu124-Gly125) is *underlined*

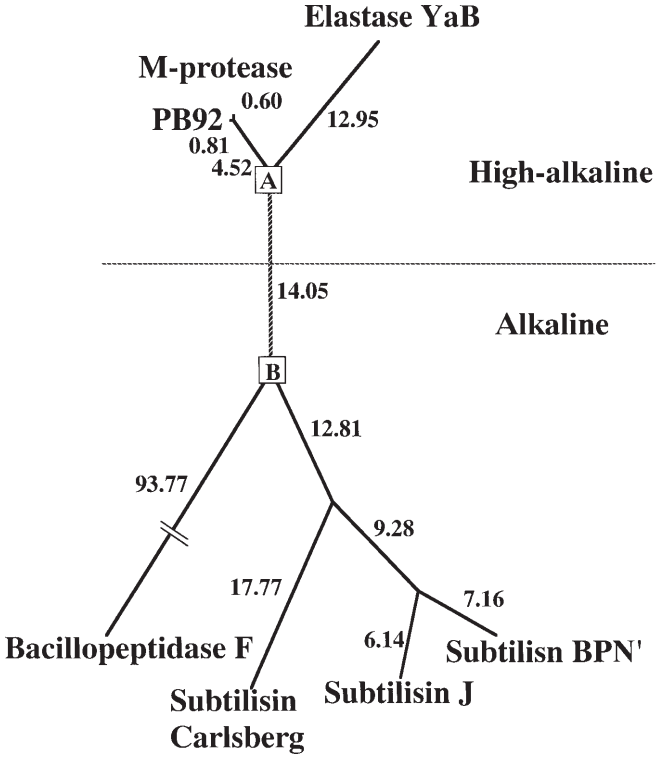

Fig. 4. Phylogenetic tree of M-protease and related alkaline proteases. Evolutionary distances (% accepted point mutations) are shown on the *branches*. The two *boxes* show positions of ancestors A and B. All branches are more than 95% probable from 1000 bootstrap reconstructions. The high-alkaline enzymes, M-protease, PB92, and elastase YaB, have pH optima for activity at around 12. The optimal pH range for less alkaline proteases, subtilisins Carlsberg, J, and BPN′, and bacillopeptidase F, is between 8 and 11

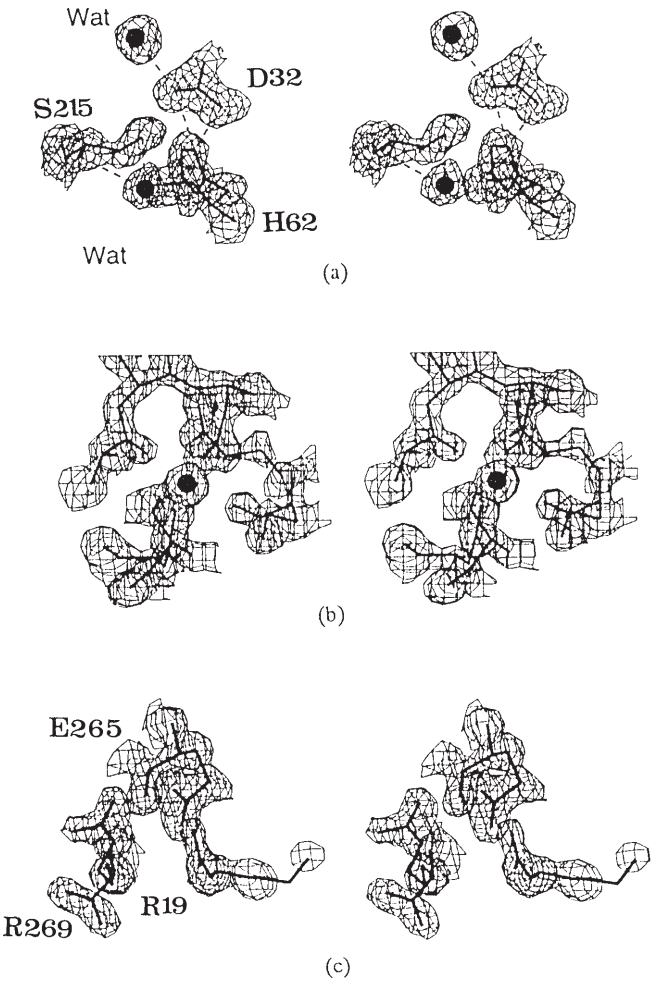

Fig. 3a–c. M-protease: the 1.6 Å elecron density map of **a** the active site; **b** the Ca-binding site; and **c** the salt bridge between Arg19, Arg269, and Glu265

proteases, and such a salt bridge is the one that is often present characteristically in the high-alkaline enzymes. The Ca-binding site is also shown in Fig. 3b.

Ancestral sequence deduced from phylogenetic tree

A molecular phylogenetic tree of high-alkaline M-protease and related less alkaline enzymes was constructed to determine the amino acid substitutions that occurred during the high-alkaline adaptation process (Shirai et al. 1997a) (Fig. 4). The total number of substitutions observed when the two ancestral sequences are compared is 42.1 residues, and the predicted number of substitutions based on the evolutionary distance (branch length between nodes A and B in the tree) is 37.4. This analysis also revealed a decrease in the number of Asp, Glu, and Lys and an increase in Agr, His, Asn, and Gln residues during the course of adaptation. Some of the aquired Arg residues form hydrogen bonds or ion pairs to combine both N- and C-terminal regions of M-

protease (see Fig. 3c). In addition, the subsituted residues were found to be localized to a hemisphere (C-terminal half) of the globular protein molecule where positional shifts of peptide segments, relative to those of the less alkaline subtilisin Carlsberg, were observed. The biased distribution and interactions caused by the substituted residues appear to be responsible for stabilization of the conformation in a high-alkaline condition.

Alkaine amylolytic enzymes from alkaliphilic *Bacillus*

Alkaline debranching enzymes are very effective to remove starch-based stains, especially when they are used in combination with α-amylase in an appropriate detergent matrix (unpublished results). We have screened and isolated a number of alkaliphilic strains of *Bacillus*, which are capable of producing alkaline debranching enzymes, such as amylopullulanase (APase), pullulanase, neopullulanase, and isoamylase.

Alkaline amylopullulanase

We found an alkaline APase in cultures of alkaliphilic *Bacillus* sp. KSM-1378 (Ara et al. 1995b). This enzyme is unique in that it efficiently hydrolyzes both the α-1,4 and the α-1,6 linkages of amylose, amylopectin, and glycogen at alkaline pH values. All the kinetic data we have obtained (Ara et al. 1995a,b) can only be explained by assuming that the dual activity of this enzyme is associated with different active sites. In fact, the alkaline APase (210 kDa) could be partially cleaved by papain to generate a 114-kDa amylose-hydrolyzing fragment and a 102-kDa pullulan-hydrolyzing fragment (Ara et al. 1996). The cloned structural gene for the APase contained a single, long ORF (5814 bp; 1938 amino acids), and the amylase and the pullulanase domains were located in the N-terminal half and in the C-terminal half of the enzyme, respectively, being separated by a tandem repeat of a sequence of 35 amino acids (Hatada et al. 1996). The APase was rotary-shadowed at a low angle and observed by transmission electron microscopy; it appeared to be a "castanet-like" or "bent dumbbell-like" molecule with a diameter of approximately 25 nm (Fig. 5). This enzyme can singly and efficiently clean difficult-to-remove starch-based stains in dishwashing and laundry detergents under alkaline conditions.

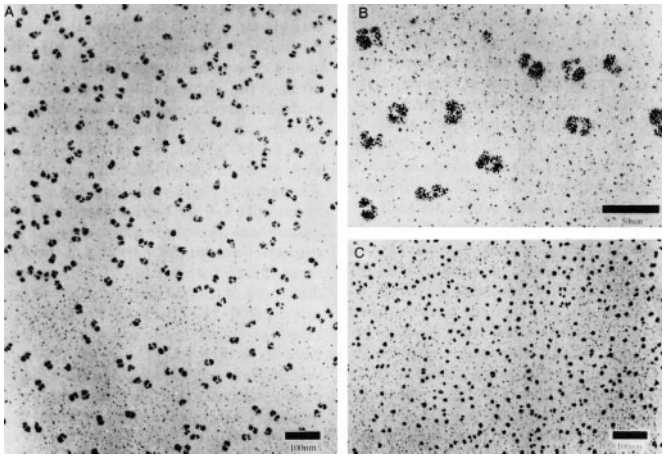

Fig. 5A–C. Transmission electron microscopic visualization of APase molecules by rotary shadowing with platinum/carbon. **A** and **B** show the native APase molecules; **C** shows a mixture of the 114-kDa amylose-hydrolyzing and the 102-kDa pullulan-hydrolyzing polypeptides generated by limited proteolysis of the native APase with papain (Ara et al. 1996). *Bar*, 100 nm (**A**,**C**) and 50 nm (**B**)

Other alkaline debranching enzymes

A novel alkaline pullulanase, found in cultures of alkaliphilic *Bacillus* sp. KSM-1876, has an optimum pH for activity of 10–10.5, which is the highest optimum pH of any pullulanases reported to date (Ara et al. 1992). The molecular mass of the enzyme was 120 kDa, as determined by SDS-PAGE. The enzyme hydrolyzed pullulan to generate maltotriose, but amylose, amylopectin, and glycogen were hydrolyzed very little, if at all. The enzyme was not inhibited at all by various surfactants and chelating reagents (EDTA, EGTA, and Zeolite). We also isolated the gene for an alkali-resistant neopullulanase from the same organism (Igarashi et al. 1992). This enzyme hydrolyzed pullulan efficiently to generate panose, and also hydrolyzed amylose and amylopectin less efficiently to generate maltose, as major end products.

An alkaline iosoamylase activity was detected in cultures of alkaliphilic *Bacillus* sp KSM-3309 (Ara et al. 1993). The enzyme had a pH optimum of 9.0, cleaved the branching points of both amylopectin and glycogen, but did not hydrolyze amylose and pullulan. Amemura et al. (1988) and Tognori et al. (1989) sequenced the gene for the isoamylase from *Pseudomonas* spp. and found that their amino acid sequences showed considerable homology to those of α-amylases and pullulanases; homology was especially conspicuous in the regions that included the proposed catalytic and substrate-binding sites. Because these pseudomonad isoamylases have optimum pH values for activity between 3 and 4, the deduced amino acid sequence of the *Bacillus* sp. KSM-3309 isoamylase may be helpful in identifying protein motifs that determine the pH optima for isoamylases of microbial origin.

Alkaline cellulases from *Bacillus*

As reviewed previously (Ito 1997; Hoshino and Ito 1997), the alkaline CMCase (941 amino acids, family A) from alkaliphilic *Bacillus* sp. KSM-635 has been incorporated into compact-type heavy-duty laundry detergents. The alkaline CMCase gene (Ozaki et al. 1990) was truncated and cloned to extract the catalytic domain. The truncated enzyme consisted of 357 amino acid residues plus 7 additional amino acid residues derived from a cloning linker,

with a predicted molecular mass of 40204 Da (Ozaki et al. 1995). Crystals of the truncated alkaline CMCase could be prepared using the hanging-drop vapor-diffusion method (Shirai et al. 1997b). The crystal belongs to trigonal space group R3, and has cell dimensions of $a = b = 111.9$ and $c = 207.1$ Å when the crystal lattice is converted into hexagonal axes. The statistical analysis is shown in Table 1. The rather high R_{merg} value may be attributed to the small crystal size.

Recently, Ducros et al. (1995) determined the crystal structure of the catalytic domain of CelCCA, which belongs to the same family as the alkaline CMCase from *Bacillus* sp. KSM-635. However, comparison of the two amino acid sequences indicates a large difference between CelCCA and the truncated alkaline CMCase in the structure of active site (amino acid identity, 17.8%). Although the active sites are conserved in the two enzymes (except for Glu307 of CelCCA, which is replaced by Asp494 in the alkaline CMCase), five (Phe42, Trp180, Trp181, Trp259, and Trp340) of the eight aromatic side chains that lie in the substrate-binding cleft of CelCCA are replaced by smaller side chains in the alkaline enzyme (Gly254, Gly380, Gly381, Ser447, and Thr522, respectively), as shown in Fig. 6. It is possible that the alkaline CMCase differs in the substrate-binding mode from CelCCA, and thus belongs to a distinct structure class in family A. Elucidation of how these substitutions alter the active site structure and how they are related to the shift in the optimum pH of the alkaline CMCase (9.5) from that of CelCCA (6.0) is of particular interest.

Table 1. Diffraction data processing statistics

Resolution range (Å)	20.0–3.3
No. of total reflections	89912
No. of independent reflections	11790
Average of I/σ(I)	4.2
Completeness (I > σ(I)) (%)	81.1
of final shell (3.42–3.30 Å)	55.3
R_{merg}[a]	0.116
of final shell (3.42–3.30 Å)	0.217

[a] $R_{merg} = \Sigma|I_i - \langle I \rangle|/\Sigma I_i$, where I_i is the scaled intensity of the *i*th observation and the <I> is the average of intensities that are equivalent to I_i.

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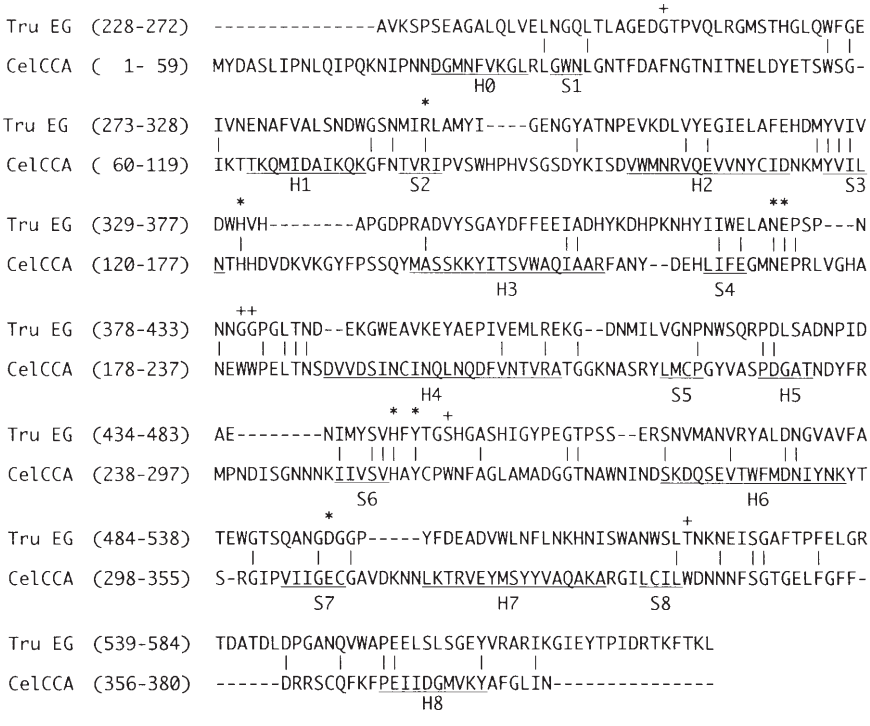

Fig. 6. Comparison of the amino acid sequence of the truncated alkaline CMCase (Tru EG) with that of the catalytic domain of CelCCA. Conserved amino acid residues are indicated by *vertical bars* between the sequences. Residues that comprise the catalytic site of CelCCA are indicated by *asterisks*. Residues denoted by *pluses* are aromatic amino acids in CelCCA and may constitute substrate-binding sites, but are replaced by smaller amino acids in the case of the truncated alkaline CMCase from *Bacillus* sp. KSM-635. Secondary structure elements are *underlined*, and labeled H0–H8 for helices and S1–S8 for strands from the N- to the C-terminal

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