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REVIEW

Detergent Alkaline Proteases: Enzymatic Properties, Genes, and Crystal Structures

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Subtilisin-like serine proteases from bacilli have been used in various industrial fields worldwide, particularly in the production of laundry and automatic dishwashing detergents. They belong to family A of the subtilase superfamily, which is composed of three clans, namely, true subtilisins, high-alkaline proteases, and intracellular proteases. We succeeded in the large-scale production of a high-alkaline protease (M-protease) from alkaliphilic *Bacillus clausii* KSM-K16, and the enzyme has been introduced into compact heavy-duty laundry detergents. We have also succeeded in the industrial-scale production of a new alkaline protease, KP-43, which was originally resistant to chemical oxidants and to surfactants, produced by alkaliphilic *Bacillus* sp. strain KSM-KP43 and have incorporated it into laundry detergents. KP-43 and related proteases form a new clan, oxidatively stable proteases, in subtilase family A. In this review, we describe the enzymatic properties, gene sequences, and crystal structures of M-protease, KP-43, and related enzymes

[**Key words:** subtilase, subtilisin, serine protease, alkaliphile, *Bacillus*, detergent enzyme]

Various enzymes are widely used in industrial fields such as detergent, food, and feed production, leather and textile processing, pharmaceutical production, diagnostics, and waste management. The largest market for industrial enzymes is the detergent industry, and alkaline enzymes such as proteases, α-amylases, cellulases, and lipases are incorporated into laundry and dishwashing detergents (1, 2). Among these enzymes, high-alkaline proteases are the most appropriate as detergents additives because they digest proteinaceous stains, such as keratin, blood, milk, and gravy on fabrics in highly alkaline detergent solution matrices. The high-alkaline proteases belong to subtilase family A, which is one of the six subtilase families (A to F) (3). A high-alkaline protease, no. 221 from *Bacillus* sp. (*Bacillus clausii*) 221, was the first enzyme from an alkaliphilic *Bacillus* to be identified (4). Since then, many high-alkaline proteases have been screened for use as detergent additives. We further found other high-alkaline proteases belonging to new clans in subtilase family A, namely, oxidatively stable proteases (OSPs; 5, 6), high-molecular-mass subtilisins (HMSs; 7, 8), and phylogenetically intermediate subtilisins (PISs), which are between the true subtilisins and the high-alkaline proteases (9, 10). These enzymes are produced extracellularly by alkaliphilic bacilli isolated from various sources such as soil, alkaline lakes, and the deep sea, and grown on proteinaceous substrates as the source of carbon and/or nitrogen (11).

HISTORY OF DEVELOPMENT OF DETERGENT PROTEASES

The idea of using proteases was first proposed by Röhm (German Patent GP283923, 1913), who incorporated pancreatic enzymes into a detergent. In the 1960s, the first detergent containing a bacterial protease appeared on the market. The enzyme used was subtilisin Carlsberg, one of the true subtilisins from *Bacillus licheniformis*. The good compatibility of the enzyme with detergent matrices greatly spurred the development of detergents with enzymes worldwide. Since the early 1980s, to accommodate the growth in environmental awareness and to reduce energy waste, detergent manufacturers have replaced phosphate with other detergent builders such as zeolite and silicates, and developed and incorporated bleach activators. New proteases that were highly alkaline and had better washing performance at low temperatures were sought. After the discovery of the high-alkaline protease no. 221 (4), variants such as Savinase (12), Maxacal (PB92) (13), NKS-21 (14), and M-protease (15) were isolated and developed for use as detergent additives.

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TABLE 1. Comparison of enzymatic properties of M-protease and KP-43

	M-protease	KP-43
Origin	*B. clausii* KSM-K16	*Bacillus* sp. strain KSM-KP43
Molecular mass	28 kDa	43 kDa
Isoelectric point	Above pH 10.6	pH 8.9–9.1
N-terminal sequence	AQSVPWGISRVQAP	NDVARGIVKADVAQ
Number of amino acids (mature)	269	434
Specific activity (casein)	127 U/mg	115 U/mg
Optimal pH (casein)	pH 12.3	pH 11–12
Optimal temperature	55°C	60°C
pH stability	pH 5–12 (55°C for 10 min)	pH 6–11 (40°C for 30 min)
Thermostability	<60°C (pH 10.5, 10 min, 2 mM $CaCl_2$)	<65°C (pH 10.5, 10 min, 2 mM $CaCl_2$)
Effects of oxidant	Sensitive	Stable
Stability to surfactants	Stable	Highly stable
Effect of fatty acid (oleic acid)	Inhibited	Not inhibited
Inhibitors	PMSF, DFP	PMSF, DFP

In addition, other high-alkaline proteases, YaB (16), AH101 (17), and AprM (18), were isolated and characterized extensively.

M-protease was isolated from alkaliphilic *B. clausii* KSM-K16 (15). We succeeded in the industrial-scale production of the enzyme for use as additives in compact heavy-duty laundry detergents using a hyperproducing mutant (1, 15). Its enzymatic properties (15, 19), gene sequence (20), and crystal structure (21, 22) were determined.

We have continued screening for new proteases having superior performance as detergent additives. Generally, subtilisins are readily inactivated by chemical oxidants because they all contain a Met residue in the vicinity of the catalytic Ser (3, 23). To improve their resistance to chemical oxidants in modern bleach-based detergent formulations, researchers replaced the Met residue with nonoxidizable amino acids (aa) by site-directed mutagenesis, but the mutation at this position reduces catalytic power significantly (24, 25). In a Japanese patent (JP 740710, 1974), Horikoshi and Yoshida reported that a subtilisin-like serine protease (E-1) from alkaliphilic *Bacillus* sp. strain D-6 is resistant to sodium perborate, one of the chemical oxidants. This encouraged us to screen for new alkaline proteases that prevent the loss of activity in several detergent formulations; consequently, several OSPs were isolated from alkaliphilic *Bacillus* strains (5). Among the OSPs, KP-43 (6), similar to E-1, was selected for use as a detergent additive. Presently, high-alkaline proteases and OSPs are incorporated into compact laundry detergents in Japan.

ENZYMATIC PROPERTIES AND GENES OF M-PROTEASE AND KP-43

M-protease from alkaliphilic *B. clausii* KSM-K16 was purified to homogeneity and its enzymatic properties were determined, as shown in Table 1. The molecular mass of the enzyme was 28 kDa as estimated by SDS–PAGE. The maximum activity against casein was observed at pH 12.3 and 55°C with a specific activity of 127 units per mg protein at pH 10. The isoelectric point was above pH 10.6. The enzyme exhibited the properties desired for a detergent enzyme, such as high stability against surfactants, high activity and stability in alkaline pH, and high activity against various proteins (15, 19).

Comparisons of the aa sequence, active site, and molecular mass of M-protease (20) with those of PB92 (26) and no. 221 (27) suggest that the primary structures of these three enzymes are quite similar, but a low similarity was found in the aa sequences between M-protease and true subtilisins BPN′ (Q44684) (58.7% identity) and Carlsberg (P00780) (59.5% identity). Nevertheless, the catalytic triad, Asp^{32}, His^{64}, and Ser^{221} (numbering in BPN′) is conserved as Asp^{32}, His^{62}, and Ser^{215} in M-protease.

The enzymatic properties of purified KP-43 of *Bacillus* sp. strain KSM-KP43 are also summarized in Table 1. KP-43 has a molecular mass of 43 kDa as determined by SDS–PAGE and a specific activity of 115 units per mg protein at pH 10.5. The isoelectric point was in the pH range of 8.9–9.1. Phenylmethyl sulfonylfluoride (PMSF) and diisopropyl fluorophosphate (DFP) inhibited the enzyme, and chelating reagents such as EDTA and EGTA did not inhibit the enzyme at all, similar to M-protease. The activity of KP-43 was observed in a wide pH range from 6 to 12. The optimum activity was at pHs 11–12. KP-43 was stable from pH 6 to pH 12 after incubation at 25°C for 24 h. The optimum temperature of 60°C is almost the same as those of reported high-alkaline proteases, and the enzyme is stable up to 65°C in the presence of calcium ions. The resistance to chemical oxidants was examined by exposure of KP-43 to a high H_2O_2 concentration (50 mM) at 30°C and at pH 10. The oxidative stability of the enzyme is clearly shown in Fig. 1. Studies of high-alkaline proteases showed that they were completely inactivated by hydrogen peroxide under the same conditions. From the enzymatic properties of KP-43, particularly its resistance to chemical oxidants and fatty acids, it seems to be superior to M-protease as a detergent enzyme.

We examined the complete KP-43 gene and its flanking regions (6). The open reading frame (ORF) in the nucleotide sequence encodes 635 aa. Figure 2 shows the deduced aa sequences of mature KP-43 and their alignment with those of related enzymes.

The N-terminal aa sequence, from Asn^{1} to Gly^{25}, deduced from the gene for KP-43 corresponded to that of the purified KP-43 from *Bacillus* sp. strain KSM-KP43 (Fig. 2). The calculated molecular mass of the mature enzyme was 45,301 Da, which is close to the 43 kDa of the native enzyme. We also sequenced the genes for other OSPs such as E-1, KSM-9860, LP-Ya, SD-521, and NP-1 (5). The deduced aa se-