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Isolation and Characterization of a Novel Thermostable Neopullulanase-Like Enzyme from a Hot Spring in Thailand

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A gene encoding a thermostable pullulan-hydrolyzing enzyme was isolated from environmental genomic DNA extracted from soil sediments of Bor Khleung hot spring in Thailand. Sequence comparison with related enzymes suggested that the isolated enzyme, designated Env Npu193A, was most likely a neopullulanase-like enzyme. Env Npu193A was expressed in *Pichia pastoris* as a monomeric recombinant protein. The purified Env Npu193A exhibited pH stability ranging from 3 to 9. More than 60% of enzyme activity was retained after incubation at 60 °C for 1 h. Env Npu193A was found to hydrolyze various substrates, including pullulan, starch, and γ-cyclodextrin. The optimal working condition for Env Npu193A was at pH 7 at 75 °C with K_m and V_{max} toward pullulan of 1.22 ± 0.3% and 23.24 ± 1.7 U/mg respectively. Env Npu193A exhibited distinct biochemical characteristics as compared with the previously isolated enzyme from the same source. Thus, a culture-independent approach with sequence-basing was found to be an effective way to discover novel enzymes displaying unique substrate specificity and high thermostability from natural bioresources.

Key words: pullulan-hydrolyzing enzyme; thermostable; neopullulanase

The α-amylase family includes several starch-converting enzymes exhibiting a diversity of substrates and product specificities.[1] Many enzymes in this family, including α-amylases, isoamylases, pullulanases, cyclomaltodextrinases, and cyclodextrin glycosyltransferase (CGTases), have been characterized and used widely for industrial purposes such as starch production, brewery, and baking. Industrial applications typically require extreme conditions of pH and temperature, hence novel amylolytic enzymes with compatible characteristics are desirable.[1]

Bacteria and fungi are the main sources for novel enzyme discovery, but only a minor proportion of them can be cultivated under standard conditions. It is estimated that more than 95% of these microbes are unculturable.[2] With advances in biotechnology, the unculturable microbes have become important targets for the discovery of novel enzymes having specific characteristics of academic and industrial interest. Molecular approaches searching for genes encoding target enzymes based on activity-based screening and PCR-based cloning have been exploited successfully in the isolation of many genes encoding potent enzymes from diverse environments, resulting in an increasing number of potent industrial enzyme candidates obtained from environmental DNA.[3–8]

One enzyme property crucial in industrial applications is thermostability. Extreme environments such as hot springs are considered promising sources for the direct isolation of thermostable enzymes. Our group has reported the biodiversity and abundance of bacteria and archaea from the Bor Khleung hot spring in Thailand, and has found that the majority (up to 80%) of the prokaryotes have not been reported elsewhere.[9] Recently, we isolated a novel cyclomaltodextrinase from this hot spring using a PCR-based cloning approach.[10] This enzyme, Env Cda13A, exhibited activity against cyclodextrins, starch, and pullulan to different degrees, with a temperature optimum of 50 °C. In this study, we isolated another novel thermostable enzyme, Env Npu193A, from the same hot spring. Although the two enzymes were isolated from the same source, they exhibited different biochemical characteristics, including substrate specificity and thermostability. The structure-function relationship of Env Npu193A with respect to other related enzymes in the neopullulanase subfamily is further discussed, demonstrating the unique distinct characteristics of this enzyme and thus the potential of the molecular approach for the discovery of novel enzymes of academic and industrial interest.

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Materials and Methods

Strains, growth conditions, and plasmids. *Escherichia coli* DH5αF′ was used in the cloning all recombinant plasmids. Bacterial cultures were grown in Luria-Bertani broth (LB) at 37 °C supplemented with ampicillin (100 μg/ml) or zeocin (25 μg/ml) where appropriate. Plasmid pTZ57R/T (Fermentas, Vilnius, Lithuania) was used for DNA cloning. Methylotrophic yeast, *Pichia pastoris* KM71 (Invitrogen, CA), was used as an expression host. Plasmid pPICZαA (Invitrogen) was used as a yeast expression vector. Yeasts were grown in YPD (Yeast Extract Peptone Dextrose medium) supplemented with zeocin (100 μg/ml) where appropriate.

Genomic DNA extraction from soil samples by SDS-based DNA extraction and purification. Soil sediments were collected from Bor Khleung hot spring in Ratchaburi Province, Thailand. Genomic DNA from soil samples was extracted by the SDS-based DNA extraction method established by Zhou *et al.*[11] The crude genomic DNA was purified with a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). The quantity and quality of purified DNA were analyzed by agarose gel electrophoresis.

Genome walking PCR. Genome walking PCR was performed as described by the manufacturer (Clontech, CA). Briefly, *Eco*RV-digested genomic DNA was ligated with the genomewalker adaptor provided. To obtain 5′ and 3′ flanking sequences of BK193, PCR was performed on adaptor-ligated genomic DNA template with BK193 gene-specific and adaptor primers. The primary PCR products, of approximately 1.3 kb (5′-walking) and 2 kb (3′-walking), were diluted and used as templates for nested PCR using nested gene-specific and adaptor primers, yielding nested PCR products of approximately 1.2 kb (5′-walking) and 1.9 kb (3′-walking) respectively. The following primers pairs were used: primary 5′-walking primers, BK193-1UP (5′-CTTGGGATCTTTTCGGAAGGCAGGGAG-3′) and AP1 (5′-GTAATACGACTCACTATAGGGC-3′); nested 5′-walking primers, BK193-2UP (5′-TCACTCGTGTCTTTTGTGGCAGGGTCAT-3′) and AP2 (5′-ACTATAGGGCACGCGTGGT-3′); primary 3′-walking primers, BK193-1DW (5′-CGCGACACCGTGGCTTGAAGGAGATGA-3′) and AP1; nested 3′-walking primers, BK193-2DW (5′-TCGCGCATGTTCGCAGATCATATCCG-3′) and AP2. To obtain the full-length open-reading frame of the Env *npu193A* gene, primers *Nde*I193 (5′-ATCTGTCATATGAAGAGAACTCATTGCATAAGCAGAGCC-3′) and *Eco*RI193 (5′-ACTGCAGAATTCAGTTTTGCTTTGTAATTAGGACGC-3′) were used. The PCR condition to obtain the Env *npu193A* gene was 1 cycle at 94 °C for 1 min, 35 cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 2 min, and 1 cycle at 72 °C for 5 min.

Molecular DNA cloning in P. pastoris. All PCR products were cloned into pTZ57R/T vector (Fermentas) and transformed into *E. coli* DH5αF′. For expression of Env Npu193A in *P. pastoris*, the corresponding gene without its signal sequence was PCR amplified using 193pic*ZXho*I (5′-GTTGGGCTCGAGAAGAGGTCGCCAGTGGTACCTGC-3′) and 193pic*ZSac*II (5′-GCACTGCCGCGGTCAGTTTTGCTTTGTAATTAGG-3′) primers (restriction sites for cloning underlined) and cloned into pPICZαA vector. As a result, the gene was fused in-frame with α-factor secretion signal of the vector. The resulting recombinant plasmid, pPICZ-BK193, was then integrated into *P. pastoris* KM71 by electroporation, as described by the manufacturer (Invitrogen). DNA sequencing to verify integration was performed by Bio Service Unit (National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand) and Macrogen (Seoul, South Korea).

Expression of Env Npu193A in P. pastoris. A single colony of recombinant *P. pastoris* was inoculated into 10 ml of YPD and incubated at 30 °C overnight with vigorous shaking. A 1-ml starter culture was transferred to a 2-liter conical flask containing 400 ml of BMGY (Buffered Glycerol-complex medium, Invitrogen), and the culture was grown overnight under the same conditions. After the culture reached an OD_{600} of 2–6, cells were harvested by centrifugation at 3,000 × *g* for 5 min, and resuspended in 100 ml of BMMY (Buffered Methanol-complex medium, Invitrogen) containing 3% methanol as an inducer. Induction was continued at 30 °C with vigorous shaking for 2 d with the addition of methanol every 24 h to yield a final concentration of 3%. Protein expression profiles were analyzed on SDS–PAGE with Coomassie brilliant blue staining. The culture medium containing secreted target enzyme was collected by centrifugation at 3,000 × *g* for 5 min.

Purification of Env Npu193A. The culture medium was concentrated by recirculating at a flow rate of 40 ml min^{-1} in a Minimate™ Tangential Flow Filtration (TFF) system (Pall, NY) with a 10 kDa-cut off Minimate™ TFF capsule. The protein was then diluted in potassium phosphate buffer, pH 6, to the initial volume, and the concentration step repeated. The whole process was repeated at least twice to replace the medium with the buffer entirely. Size exclusion chromatography was carried out on an AKTA Explorer system (GE Healthcare Biosciences, Uppsala, Sweden) to purify Env Npu193A. Concentrated protein sample (300 μl) was injected into a Superdex 200 10/300 GL column (GE Healthcare) pre-equilibrated with 50 mM MOPS, pH 7, and 50 mM NaCl and eluted in the same buffer at a flow rate of 0.5 ml/min. Fractions exhibiting enzymatic activity were collected and concentrated using an Amicon Ultra-15 device (Millipore).

Enzyme assay. The enzyme activity was determined

quantitatively by the DNS method.[12] Starch (0.5%) (Carlo Erba, Rodano, Italy), 0.5% pullulan or 0.5% cyclodextrin (Sigma, St. Louis, MO) substrates was incubated with appropriate amounts of enzyme in 50 mM MOPS buffer, pH 7.0, supplemented with 2 mM of $CaCl_2$. The reaction was incubated at 75 °C for 45 min and then 0.3 ml of DNS reagent was added. The reaction was then boiled for 10 min. The amount of reducing sugar was determined by measuring the absorbance at 540 nm (OD_{540}) and interpolating from a standard curve of glucose or maltotriose (for pullulanase activity) absorbance. The effect of metal ions on enzymatic activity was examined after pre-incubation of the enzyme solution with 2 mM of various cations (Mg^{2+}, Ca^{2+}, Sr^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, Mn^{2+}) at optimal temperature for 45 min, and then the remaining activity was determined as described above. One unit of enzyme activity was defined as the amount of the enzyme that produces 1 μmole of reducing sugar at the optimal temperature in 1 min. The enzyme activities measured at different intervals were confirmed to be within the linear range of the assay. The thermostability of the enzyme was determined by pre-incubating the enzyme mixture (appropriate amounts of Env Npu193A in 50 mM MOPS, pH 7.0, and 2 mM $CaCl_2$) at 45, 50, 60, 65, 70, and 75 °C for 30, 60, and 120 min. To determine pH stability, the enzyme was incubated at 25 °C for 4 h in 50 mM sodium citrate (pH 3), 50 mM sodium acetate (pH 5), 50 mM Tris–HCl (pH 7), or 50 mM glycine (pH 9). The residual pullulanase activity was measured by the DNS method using 4% (w/v) pullulan as substrate under optimum conditions. The relative activity was calculated by comparing the activity after each treatment to that of the untreated enzyme, which was assigned to be 100%. The kinetic parameters of the enzyme were calculated by fitting initial velocities measured at various substrate concentrations to the Michaelis-Menten equation using Kaleida Graph version 3.51 data analysis software (Synergy Software, PA).

Product specificities of Env Npu193A. Hydrolysis products were analyzed by thin layer chromatography (TLC). An appropriate amount of the substrate (cyclodextrins, soluble starch, pullulan (0.5% each) and 2 mM maltooligosaccharides) was incubated with the enzyme at 75 °C for 24 h. The reactions were heated at 100 °C for 10 min to stop the reaction, and spotted onto a silica gel 60 aluminum plate (Merck, Darmstadt, Germany). The plate was then placed in a TLC chamber containing a solvent mixture of isopropanol-ethyl acetate-water (3:1:1, v/v/v), and developed at room temperature. After air-drying, the detection reagent (0.3% of *N*-(1-naphthyl)-ethylenediamine and 5% concentrated H_2SO_4 in methanol) was evenly sprayed onto the plate. The plate was heated at 110 °C for 10 min for visualization.

Sequence analysis. The signal peptide cleavage site was analyzed by SignalP 3.0.[13] Multiple sequence alignments were performed using the program Clustal X.

Results

Identification of a novel gene encoding a neopullulanase-like enzyme by genome-walking PCR

Previously, we reported the identification of partial gene sequences with homology to the conserved region of the starch-degrading enzyme family, and one of these sequences, BK13, was further characterized.[10] The full-length gene of BK13, designated Env *cda13A*, was identified by a genomic walking approach, and the biochemical properties of the corresponding enzyme were investigated.[10] In this work, the same approach was employed to obtain a full-length gene of another partial sequence, BK193, previously isolated from the same hot-spring environmental DNA sample. The full-length gene, designated Env *npu193A*, was 1,815 bp in length (GenBank accession EU022022) and coded for a putative protein of 605 amino acid residues. BLAST analysis showed that Env Npu193A shared the highest amino acid sequence identity (48%) to that of Env Cda13A, while it exhibited 45% identity with α-amylase of *Salinibacter rubber* DSM13855 and 37% identity with chain B of α-amylase II (TVAII) of *Thermoactinomyces vulgaris*. The alignment of Env Npu193A with related enzymes is shown in Fig. 1, and salient conserved features are highlighted.[1] The neopullulanase subfamily conserved regions are shown as boxes A-C in Fig. 1.[14–16] Box A locates between the start of the $(\beta/\alpha)_8$-barrel and conserved region I, which is perhaps a Ca^{2+}-binding site.[17] The conserved sequences in BoxA and BoxB (HKYXT and YQIFPER) are absent in pullulan non-hydrolyzing amylases. Box C denotes the fifth conserved region, used to distinguish between oligo-1,6-glucosidase subfamily (QpDln) and neopullulanase subfamily (MpKln), and is perhaps involved in calcium binding.[16] The amino acid sequences in BoxC of Env Npu193A are different from both subgroups, but are similar to that of cyclomaltodextrinase of *Thermotoga maritima* and Env Cda13A.[10,15] In addition, these latter three enzymes lack an extended N-terminal domain, which is the unique characteristic of true members of the neopullulanase subfamily. Env Npu193A does not contain the consensus sequence YNWGYDP, which is found in all type I pullulanases.[18] Therefore, Env Npu193A can be included in the group of so-called neopullulanse-like enzymes.[16]

Enzyme characterization

Env Npu193A contained a putative signal sequence of 27 amino acid residues at the N-terminus (M_1-Q_{27}) (Fig. 1). This indicates that it is an extracellular protein. It was expressed as a fusion protein (residues 28 to 605) with the α-factor peptide, and secreted as an active soluble protein in *P. pastoris* with an apparent MW of approximately 66 kDa (Fig. 2). Purification of Env

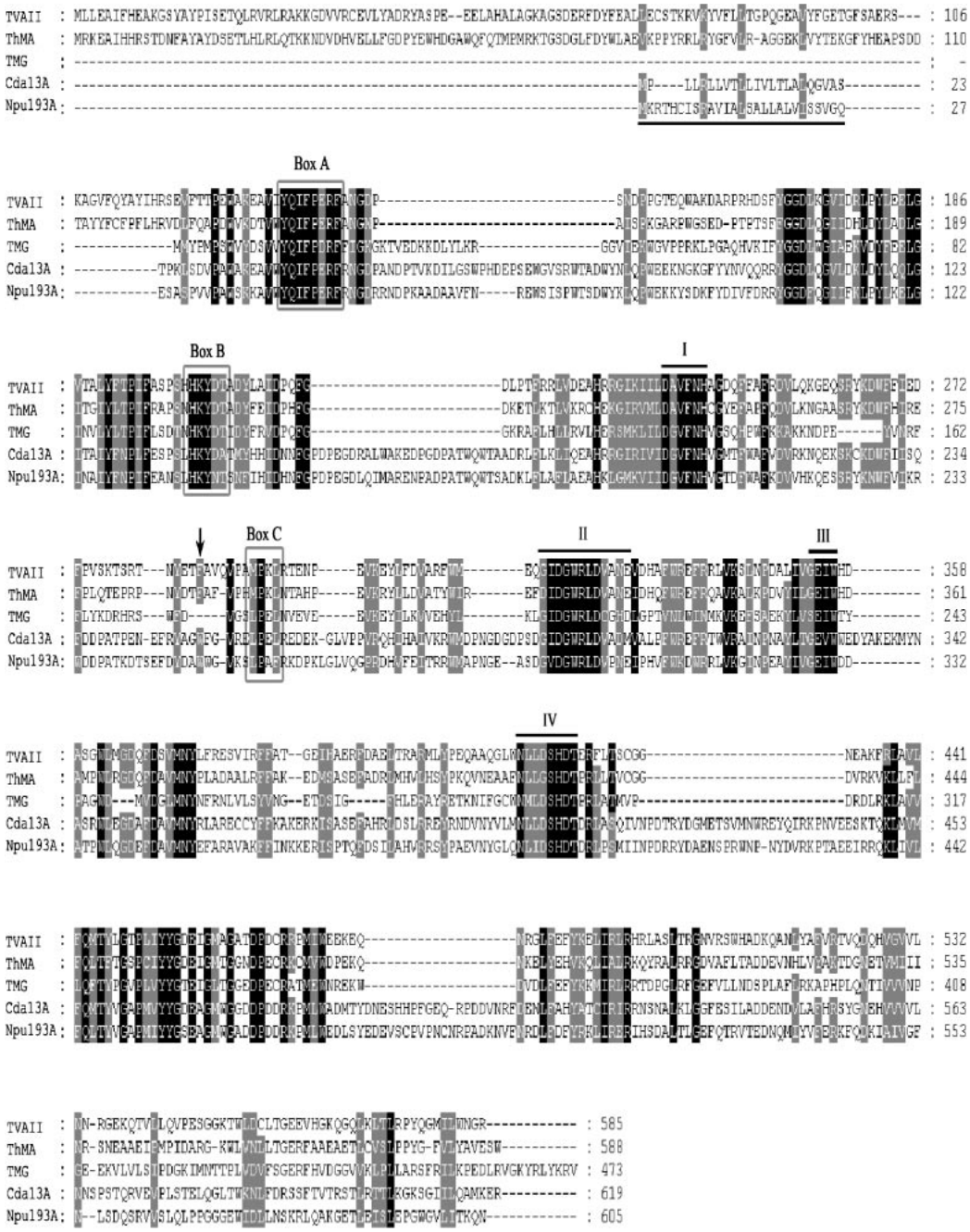

Fig. 1. Multiple Sequence Alignment of Env Npu193A Isolated from Hot Spring with Other Related Amylolytic Enzymes.

The four conserved regions commonly found among enzymes in glycosyl hydrolase family 13 are shown (labeled as I-IV). Conserved regions located between the start of the $(\beta/\alpha)_8$-barrel and conserved region I are indicated as BoxA and BoxB. The fifth conserved region, proposed to be a selection marker for the neopullulanase subfamily, is indicated by BoxC.[16] The putative signal sequence of Env Npu193A is underlined. The Phe residue corresponding to Phe289 of *Thermus* sp. is indicated by an arrow. TVAII, chainB of α-amylase II of *Thermoactinomyces vulgaris* (accession no. BAB40638); ThMA, maltogenic amylase of *Thermus* sp. IM6501 (accession no. AAC15072); TMG, maltogenic amylase of *Thermotoga maritima* (accession no. AE001820); Cda13A, cyclomaltodextrinase Env Cda13A (accession no. DQ191050); Npu193A, neopullulanase-like enzyme Env Npu193A (accession no. EU022022).

Npu193A by gel filtration revealed a monomeric protein of similar size (data not shown). The purified enzyme exhibited highest activity toward pullulan (23.24 U/mg), and showed lower activity against starch (0.15 U/mg). It was able to hydrolyze γ-cyclodextrin, but with very poor activity (data not shown). Ca^{2+} was essential for the activity of the enzyme, since no activity was detected when Ca^{2+} was not included in the reaction (data not shown). The substrate specificity corresponded well with the fact that Env Npu193A functioned extracellularly, since it prefers large substrates that cannot enter the cells. Since the enzyme showed highest activity toward pullulan, it was chosen as a substrate to investigate further the properties of the Env Npu193A enzyme. The

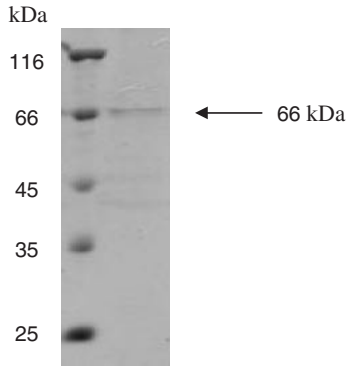

Fig. 2. Expression of Env Npu193A in *P. pastoris*.
Culture was induced for 2 d with methanol, as described in "Materials and Methods," and the supernatant from day 2 was collected and subjected to SDS–PAGE.

recombinant enzyme showed optimal temperature and pH of 75 °C and 7 (Fig. 3A and B). The K_m and V_{max} values for pullulan were $1.22 \pm 0.3\%$ and 23.24 ± 1.7 U/mg respectively. The high enzymatic activity toward pullulan of Env Npu193A was different from that of the previous isolate, Env Cda13A, from the same environment, which preferred cyclodextrins as substrates (23.24 U/mg for Env Npu193A as compared with 0.3 U/mg for Env Cda13A when pullulan was used as a substrate). Env Npu193A was stable across broad pH range, from 3 to 9, with at least 50% remaining activity (Fig. 4A). In addition to its ability to function at high temperatures, Env Npu193A exhibited high thermostability as compared with Env Cda13A. After incubation at 60 °C for 60 min, it retained more than 80% of maximal activity (Fig. 4B) whereas Env Cda13A activity was completely abolished under these conditions (data not shown).

Hydrolysis products of Env Npu193A

Products obtained from Env Npu193A-catalysed hydrolysis were analyzed by TLC (Fig. 5). The results showed that this novel enzyme hydrolyzed large molecules of pullulan, starch, and γ-cyclodextrin, but not α or β-cyclodextrins (Fig. 5A). The enzyme also hydrolyzed linear maltooligosaccharides (G4-G6) (Fig. 5B). The final products obtained from complete Env Npu193A hydrolysis of starch, γ-cyclodextrin, and malto-oligosaccharides were maltose, but not glucose. Pullulan degradation by Env Npu193A yielded only maltotriose, not panose. This suggests that this enzyme exhibited activity only on α-1,6-glucosidic linkages of pullulan, not on α-1,4-glucosidic bonds. This supports the assumption that Env Npu193A is not a true member of the neopullualnase subfamily, which, by definition, hydrolyses α-1,4-glucosidic linkages in pullulan yielding panose. Some are also able to hydrolyze α-1,4-glucosidic and α-1,6-glucosidic linkages in pullulan.[18]

Discussion

Enzymes belonging to the neopullulanase subfamily typically harbor the N-domain, which is involved in dimer formation.[14,19] In addition, amino acids at the extended N-terminal domain of enzymes in the neopullulanase subfamily have been found to play roles in cyclodextrin binding, although those roles are not essential.[15,19] It was found that enzymes lacking this N-domain were still able to hydrolyze cyclodextrins, but with less activity than those harboring this region.[20] This lack of N-domain corresponded well with Env Npu193A, which is monomeric, and the low activity on cyclodextrins of both Env Cda13A and Env Npu193A.[10] The crystal structure of a maltogenic amylase (ThMA) of *Thermus* sp. revealed three residues in the N-terminal domain, Tyr45, Trp47, and Asp110, which interacted with β-cyclodextrin at the upper part of the active site.[15] Another amino acid, Phe286, is responsible for interaction with β-cyclodextrin. Replacement of Phe286 by non-aromatic amino acid residues causes a drastic decrease in the k_{cat}/K_m values, due to the loss of interaction between the aromatic ring of Phe and the ring structure of β-cyclodextrin.[21] For both Env Cda13A and Env Npu193A, this Phe is replaced by Trp (Fig. 1). Although the two enzymes share the same aromatic residue, which should make for similar interactions with cyclodextrins, our result indicates that the cyclodextrin substrates were converted more efficiently by Env Cda13A than Env Npu193A. This clearly indicates that other amino acid residues also contribute to substrate recognition in the neopullulanase subfamily, resulting in the clear difference in substrate specificity of these two enzymes.

Recently, there was a study of mutagenesis of a conserved residue Ala290 of a maltogenic amylase, ThMA, from *Thermus* sp. IM6501.[22] Replacement of this amino acid residue with one with a bulky side chain resulted in a change in substrate specificity of the enzyme. The mutant, ThMA-A290I, produced more maltose than glucose, while the wild-type enzyme produced both sugars in comparable amounts. It was found that the mutation at this position increased the K_m value for maltose, resulting in an accumulation of this sugar.[22] Concomitantly, the presence of Trp residue at the corresponding position (W251) of Env Npu193A is likely to be the reason maltose was the main product obtained from starch and cyclodextrin hydrolysis by the enzyme. The bulky side chain of Trp residue might affect the substrate binding affinity in such a way that maltose was not further hydrolyzed to glucose. Env Cda13A, in contrast, hydrolyzed various substrates, yielding glucose as the main product.[10] Since Env Cda13A harbored Phe at the corresponding position, it is possible that the less bulky side chain of Phe residue allows higher affinity for maltose binding, leading to more glucose as the product as compared with Env Npu193A.

In this study, the preferred substrate for Env Npu193A

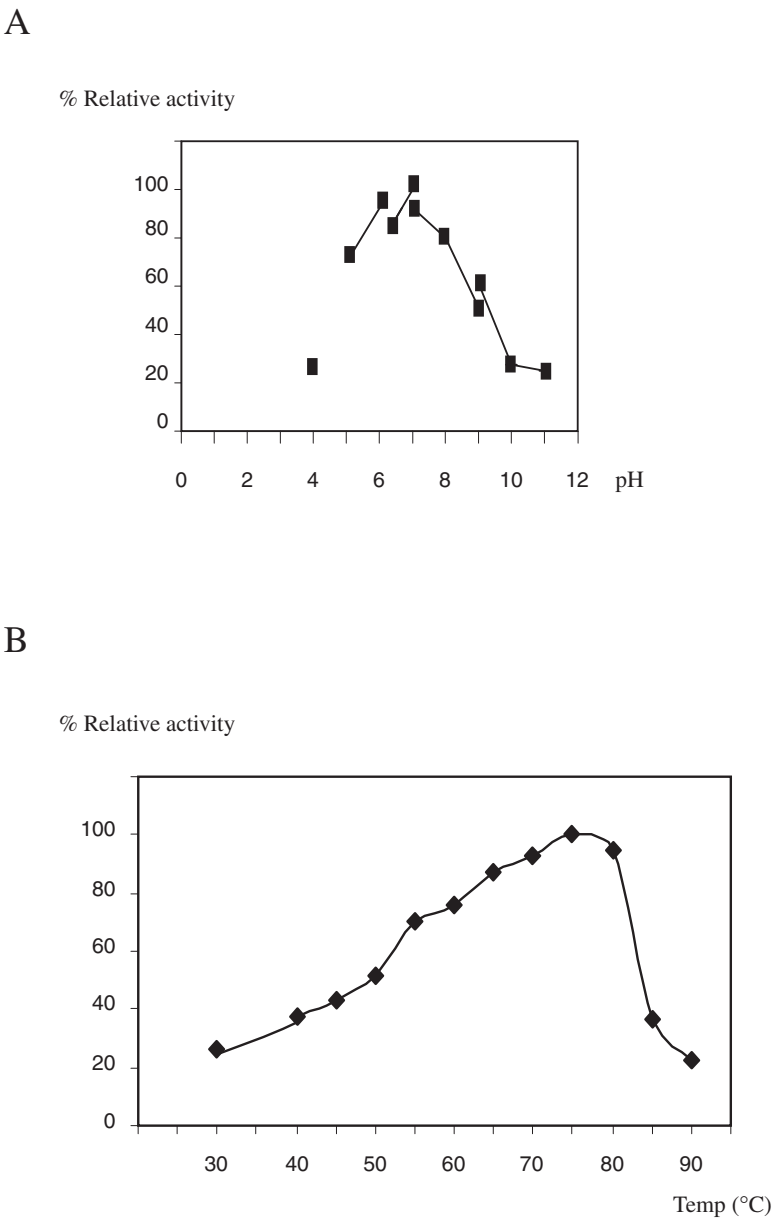

Fig. 3. Activity Profiles of the Purified Env Npu193A.

The enzyme activities were measured at various pH (A) and various temperatures (B). Optimal pH was determined by measuring the enzyme activity in buffer ranging from 4–11 (100 mM): sodium citrate (pH 4), acetic acid (pH 5–6), MOPS (pH 6.5–7), Tris–HCl (pH 7–9), and Glycine (pH 9–11). The optimal temperature was determined at selected temperatures from 30 to 90 °C.

was pullulan, which was hydrolyzed only to maltotriose. The involvement of conserved region II for substrate specificity among starch-hydrolyzing enzymes has been demonstrated with TVAI α-amylase from *T. vulgaris*.[23] Substitution of amino acid sequences, AAQY, at the C-terminal of conserved region II in this enzyme into VANE resulted in the alteration of substrate specificity. The mutant TVAI enzyme hydrolyzed mainly the α-1,6-glucosidic bond of pullulan, yielding maltotriose, whereas the action patterns for the other substrates remained the same as the wild type.[23] Mutation in conserved region II caused the alteration of the k_{cat}/K_m value of the enzyme on α-1,4-glucosidic linkages.[23] This might explain the preference for pullulan of Env Npu193A, which contains the sequence VPNE in conserved region II (Fig. 1). On the other hand, Env Cda13A with VADM sequences and the cyclodextrinase of *T. maritima* harboring four completely different amino acid residues (CGHD) in this conserved region degraded pullulan poorly (Fig. 1).[15] A pullulan hydrolase from *Thermococcus aggregans* that attacks both the α-1,4-glucosidic and the α-1,6-glucosidic bonds in pullulan has a hybrid sequence in this region (APQE).[24] This enzyme has been proposed to be a pullulan hydrolase type III.[18] This indicates the important role of amino acid sequences in conserved region II for interaction and

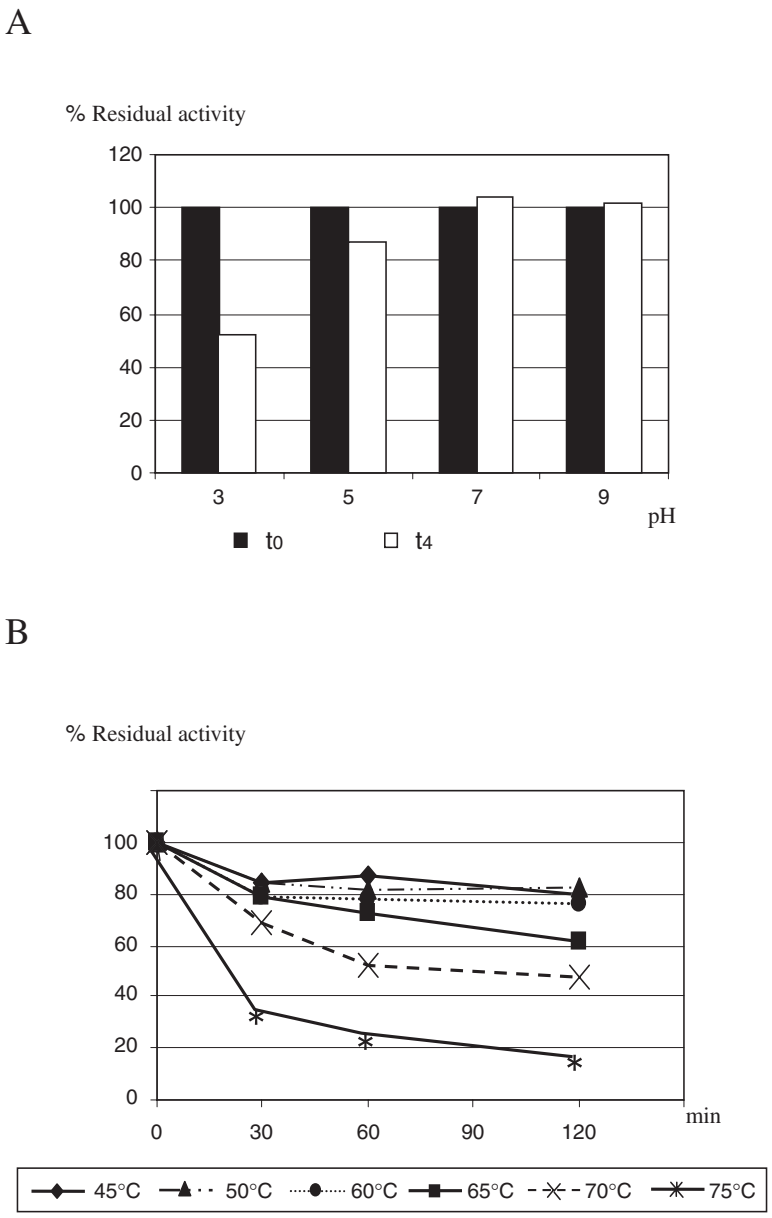

Fig. 4. Effect of pH (A) and Temperature (B) on the Stability of Env Npu193A.

To assess the pH stability of Env Npu193A, the enzyme was pre-incubated at 25 °C for 4 h in 50 mM sodium citrate (pH 3), 50 mM sodium acetate (pH 5), 50 mM Tris–HCl (pH 7), and 50 mM glycine (pH 9) before it was mixed with the reaction mixture, as described in "Materials and Methods." ■, Enzyme activity from Env Npu193A that was not pre-incubated; □, Enzyme activity from Env Npu193A after 4 h of pre-incubation. The thermostability of the enzyme was determined by pre-incubating Env Npu193A for 30, 60, and 120 min at various temperatures before it was mixed with the reaction mixture. Residual pullulanase activity was measured by the DNS method. Values are shown as percentages of the original activity, which was taken to be 100%. Each data plot is the average of two.

reaction specificity on pullulan. In conserved region III (EIWD), the Ile residue has been found to play an important role in the substrate specificity of neopullulanase.[25] Replacement of this amino acid residue with Trp (I358W) caused the neopullulanase of *B. stearothermophilus* to hydrolyze starch rather than pullulan. On the other hand, the I358V mutation increased the specificity of neopullulanase for α-(1,6)-branched oligosaccharides. Therefore, it is proposed that this Ile residue in conserved region III affected the substrate preference of the enzyme.

In summary, the neopullulanase-like enzyme Env Npu193A is capable of functioning at high temperatures and of exhibiting high thermostability. It displays different biochemical characteristics to Env Cda13A, previously isolated from the same source, and many related enzyme previously reported. The unique properties of Env Npu193A indicate the potential of culture-independent methods to retrieve such novel enzymes from natural bioresources. The sequence-based screening approach can be used to discover new genes that might go undetected using expression-based screening.

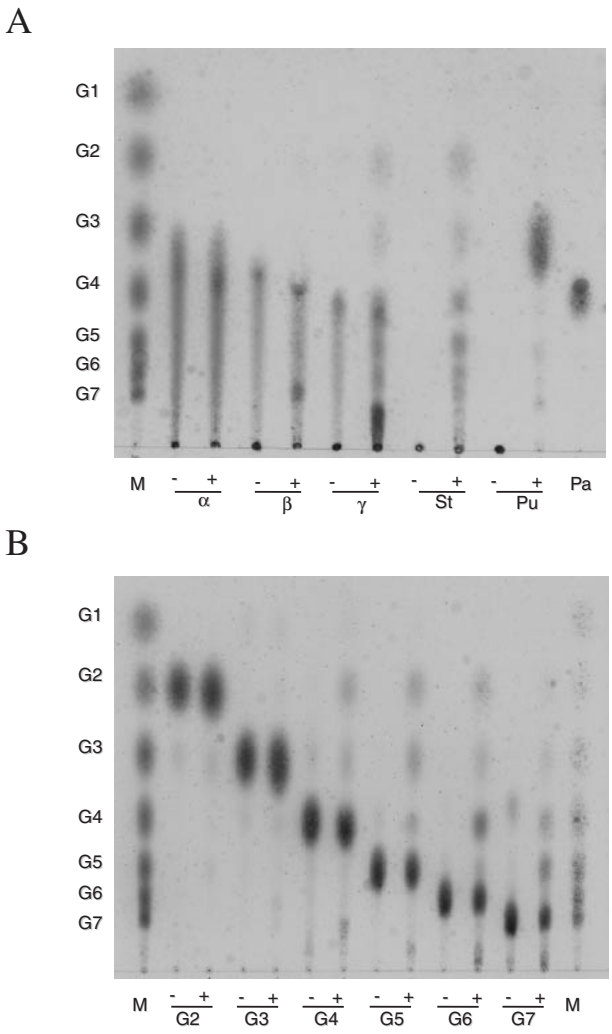

Fig. 5. Product Specificity Profiles of Env Npu193A.

A, cyclodextrins: (α-CD, β-CD, γ-CD); soluble starch (St), and pullulan (Pu). B, malto-oligosaccharides: maltose (G2); maltotriose (G3); maltotetraose (G4); maltopentose (G5); maltohexose (G6) and maltoheptaose (G7). Reactions with and without the enzyme are indicated by + and − respectively. The molecular weight standard (M) was a mixture of oligosaccharides from glucose (G2) to maltoheptaose (G7). Pa was a standard panose.

Therefore, this approach is useful in retrieving novel enzymes that are not able to express actively under the limited hosts and expression systems used or the detection methods for such enzymes under those conditions. Isolation of genes for novel enzymes from unculturable microbial resources has yet again proved to be an attractive approach to finding potent enzymes of academic and biotechnological interest.

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