Title

Truncation of N- and C-terminal regions of *Streptococcus mutans* dextranase enhances catalytic activity

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Abbreviations

AP, antisense primer; CI, cycloisomaltooligosaccharide; CITase, CI glucanotransferase; CR, catalytic region; C-VR, C-terminal variable region; Dex, endo-dextranase; ESI-MS, electrospray ionization-mass spectrometry; GBS, glucan-binding site; GH, glycoside hydrolase family; IG2 to IG7, isomaltooligosaccharides having 2–7 glucose units, respectively; N-VR, N-terminal variable region; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; pNP-IG3, *p*-nitrophenyl α-isomaltotrioside; pNP-IG4, *p*-nitrophenyl α-isomaltotetraoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SmDex, dextranase from *Streptococcus mutans* ATCC 25175; SmDex58, SmDex70, SmDex90 and SmDex95, enzymatically active SmDexs with 58.1 kDa, 69.7 kDa, 89.8 kDa and 95.4 kDa, respectively; SP, sense primer; TM, truncation mutant of SmDex.

Abstract Multiple forms of native and recombinant endo-dextranases (Dexs) of the glycoside hydrolase family (GH) 66 exist. The GH 66 Dex gene from *Streptococcus mutans* ATCC 25175 (SmDex) was expressed in *Escherichia coli*. The recombinant full-size (95.4 kDa) SmDex protein was digested to form an 89.8 kDa isoform (SmDex90). The purified SmDex90 was proteolytically degraded to more than seven polypeptides (23–70 kDa) during long storage. The protease-insensitive protein was desirable for the biochemical analysis and utilization of SmDex. GH 66 Dex was predicted to comprise four regions from the N- to C-termini: N-terminal variable region (N-VR), catalytic region (CR), glucan-binding site (GBS), and C-terminal variable region (C-VR). Five truncated SmDexs were generated by deleting N-VR, GBS and/or C-VR. Two truncation-mutant enzymes devoid of C-VR (TM-NCGΔ) or N-VR/C-VR (TM-ΔCGΔ) were catalytically active, thereby indicating that N-VR and C-VR were not essential for the catalytic activity. TM-ΔCGΔ did not accept any further protease-degradation during long storage. TM-NCGΔ and TM-ΔCGΔ enhanced substrate hydrolysis, suggesting that N-VR and C-VR induce hindered substrate binding to the active site.

Key words endo-dextranase · glycoside hydrolase family 66 · limited proteolysis · truncation

Introduction

Endo-dextranases (Dexs; EC 3.2.1.11) randomly hydrolyze the internal α-1,6-linkages of dextran. Dexs are classified into two groups [glycoside hydrolase family (GH) 49 or 66] on the basis of similarities in their amino-acid sequences (Henrissat and Bairoch 1996). GH 66 consists of Dex (Barrett et al. 1987; Lawman and Bleiweis 1991; Wanda and Curtiss 1994; Igarashi et al. 1995a, 1995b, 2001, 2004a) and cycloisomaltooligosaccharide glucanotransferase (CITase; EC 2.4.1.248) (Oguma et al. 1995). CITase synthesizes the cycloisomaltooligosaccharide (CI; cyclic saccharide linked by α-1,6-glucosyl units) from dextran with intramolecular transglycosylation, which mainly produces CI-8 (CI with eight glucosyl units) (Oguma et al. 1993, 1994). Alignment of the amino-acid sequences of GH 66 Dexs (Fig. 1) demonstrated that the enzymes comprise of four regions from the N- to the C-termini: the N-terminal variable region (N-VR), conserved region (CR), glucan-binding site (GBS) and C-terminal region (C-VR) (Igarashi et al. 2002, 2004a; Morisaki et al. 2002). Recently, Yamamoto et al. (2006) reported that two Asp residues of CITase (Asp145 and Asp270) were crucial for enzymatic activity. These functionally important Asp residues are located at CR of Dex. Although the three-dimensional structures of GH 66 enzymes are not available, it was predicted that CR of GH 66 Dexs forms a catalytic $(\beta/\alpha)_8$ barrel, as has been observed in GH 13, 27 and 31 proteins (Rigden 2002), and all of which probably share a common evolutionary origin. Aoki and Sakano (1997) reported that only CITase has a long insertion of 89 amino acids inside a CR of Dex (Fig. 1), which might relate to the unique intramolecular transglycosylation activity of CITase.

Formation of protease-associated multiple isoforms has been reported on many GH 66 Dexs of native enzymes and recombinant enzymes (Khalikova et al. 2005). Some degraded polypeptide fragments still maintain the dextranolytic activity. Expression of Dex gene of *Streptococcus sobrinus* (which encodes a 143 kDa protein) using *Escherichia coli* produced six forms varying from 125 to 170 kDa (Wanda and Curtiss 1994). Similar observations were also reported on recombinant Dex of *Streptococcus mutans* Ingbritt (four isoforms) (Igarashi et al. 1995a), *Streptococcus salivarius* (three isoforms) (Lawman and Bleiseis 1991) or *Streptococcus downei* (several isoforms) (Igarashi et al. 2001). Finnegan et al. (2004a, 2004b, 2005) isolated 11 strains of *Paenibacillus*, all of which secreted 2–5 Dexs into the culture medium. Native Dex from *Bacillus* sp. was digested at its C-terminal region during cultivation, resulting in three isoforms (Khalikova et al. 2003). Although GH 66 Dexs are frequently degraded by protease, no isoform has yet been purified, blunting knowledge of the effect of proteolytic digestion on catalytic activity. Since proteolytic degradation normally results in decreased enzyme activity and stability, protection against protease attack is desirable for the industrial application of Dexs.

Genomic DNA of *S. mutans* ATCC 25175 contains a gene encoding GH 66 Dex (SmDex). SmDex is composed of five portions (Fig. 1): a signal peptide sequence (N-terminal 24 amino acids), N-VR (Ser25 to Asn99), CR (Gln100 to Ala615), GBS (Leu616 to Ile732), followed by V-CR (Asn733 to Asp850). By our demand for a large amount of short isomaltooligosaccharides, we expressed an SmDex gene using *E. coli* and examined the activity of the resulting recombinant enzyme with dextran. During this study, the produced full-size SmDex having Ser25-Asp850 with 95 kDa (SmDex95) was digested

with protease to form many polypeptides. To obtain a protease-resistant SmDex, the protease-sensitive region was removed from SmDex by a truncation approach to construct a series of truncation mutants (TMs): TM-NCGΔ (deleting C-VR), TM-NCΔΔ (deleting GBS/C-VR), TM-ΔCGΔ (deleting N-VR/C-VR), TM-ΔCΔΔ (deleting N-VR/GBS/C-VR), and TM-ΔCsΔΔ (deleting N-VR/C-VR; having a short CR of Gln100 to Leu611). The resulting truncated enzymes of TM-NCGΔ and TM-ΔCGΔ displayed the enhanced substrate hydrolysis, clarifying the possible function of N-VR and C-VR.

Materials and methods

Materials

All chemicals were of analytical grade. Solvents were purchased from Kanto Chemical (Tokyo, Japan); dextran (T10 and T2000) and blue dextran 2000, from Amersham Pharmacia Biotech (Uppsala, Sweden); isomaltooligosaccharides having 2–7 glucose units (IG2–IG7, respectively), from Seikagaku (Tokyo, Japan); phenylmethylsulfonyl fluoride (PMSF), from Nakarai Tesque Chemical (Kyoto, Japan); *S. mutans* ATCC 25175, from the American Type Culture Collection (Manassas, VA, USA); restriction endonucleases, from Takara Bio (Otsu, Japan); primers used for polymerase chain reaction (PCR), from Sigma-Aldrich Japan (Tokyo, Japan). *p*-Nitrophenyl α-isomaltotrioside (pNP-IG3) and *p*-nitrophenyl α-isomaltotetraoside (pNP-IG4) were synthesized from IG3 and IG4, respectively (Trevelyan 1996; Hakamada et al. 2000). CI-8 was a gift from Dr. T. Oguma, Kikkoman, Noda, Japan.

Cloning of SmDex gene and construction of SmDex95 expression vector

S. mutans ATCC 25175 was cultivated in Brain-Heart Infusion medium (Becton and Dickinson, Sparks, MD, USA) for 2 days under anaerobic conditions at 37°C. The SmDex gene was PCR-amplified using genomic DNA of *S. mutans* ATCC 25175 and two primers designed according to the *dexA* sequence of *S. mutans* UA159 (Igarashi et al. 1995b): sense

primer (SP) [5'–(-10)CGCTGAGCATATGGAACAGTCAAATAG(17)–3' having underlined *Nde*I site (numbers indicate the nucleotide positions from A of initial ATG); designated SMU_1] and antisense primer (AP) [5'–(2553)TCAATCATTTTTTCTACCCTTTATC(2528)–3']. The amplified fragments were inserted into *Eco*RV site of pBluescript II SK(+) vector (Stratagene, La Jolla, CA, USA), resulting in an *SmDex95*-pSK plasmid. The generated plasmid was sequenced using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The amplification and sequencing of the SmDex gene were repeated twice to confirm the sequence data.

To construct an expression plasmid of SmDex95 (full length SmDex without a signal peptide) with His6-tag at the N- and C-termini (Fig. 2), the SmDex gene was amplified using *SmDex95*-pSK and the primers of SMU_1 (harboring an *Nde*I-restriction site) and AP [5'–(2550)CACTATTGCTCGAGATCATTTTTTCTACC(2538)–3' having an underlined *Xho*I site]. The resulting PCR product and pET28a (Novagen, Madison, WI, USA) were digested with *Nde*I and *Xho*I, and ligated using the DNA Ligation Kit ver. 2 (Takara Bio). The resulting plasmid, designated *SmDex95*-pET28a, was sequenced with an automated DNA sequencer.

Construction of vectors for expressing 5 kinds of TMs

Construction of pET28a-derived expression vectors for five TMs with two His6-tags at their N- and C-termini (Fig. 2) followed the aforementioned procedure, unless mentioned

otherwise. For TM-ΔCsΔΔ, PCR was done using SP [5'–(283)GAAACTGCCATGGATCAAAAGAATGGC(309)–3' having underlined *Nco*I site], AP [5'–(1122)CATAGCTTGAGCCATTGCATTAGAAAT(1096)–3'], and *SmDex95*-pET28a. The resulting 0.8 kb fragment was used for megaprimer PCR (Datta 1995) with AP [5'–(1848)CAAAGCATCTTGCTCGAGCTGCCGACC(1822)–3' having underlined *Xho*I site] and *SmDex95*-pET28a. The 1.5 kb amplified product and pET28a were digested with *Nco*I and *Xho*I and ligated to generate the *TM-ΔCsΔΔ*-pET28a expression plasmid. For TM-ΔCΔΔ, PCR was done using SP [5'–(494)ATACAACTCAAAAAGGTGAATTCGTT(519)–3'; designated SMU_2], AP [5'–(1860)GCCATTGATCTCGAGAGCATCTTGCGA(1834)–3' having underlined *Xho*I site], and *SmDex95*-pET28a. The 1.4 kb amplified product was digested with *Bsp*HI (New England Biolabs, Beverly, MA, USA) and *Xho*I, followed by insertion into the *Bsp*HI-*Xho*I site of *TM-ΔCsΔΔ*-pET28a, resulting in plasmid *TM-ΔCΔΔ*-pET28a. For TM-ΔCGΔ, the PCR with SP [5'–(2180)GGGATATGATTTATATTCTCGAGACCATT(2208)–3' having underlined *Xho*I site], AP [5'–CACTATTGCTCGAGATCATTTTTTCTACC(2536)–3' having underlined *Xho*I (CACTATTGCTC at 5'-end was a sequence of pET28a)] and *SmDex95*-pET28a generated a 0.4 kb DNA fragment that was used for megaprimer PCR with SMU_2 and *SmDex95*-pET28a. The 2.1 kb amplified product and *TM-ΔCsΔΔ*-pET28a were digested with *Eco*RI and *Xho*I and ligated to form plasmid *TM-ΔCGΔ*-pET28a. For TM-NCΔΔ and TM-NCGΔ, *TM-ΔCsΔΔ*-pET28a and *TM-ΔCGΔ*-pET28a, respectively, was utilized. The two plasmids were digested with *Eco*RI and *Xho*I. Each fragment was introduced to the same restriction sites of *SmDex95*-pET28a, generating plasmid

TM-NCΔΔ-pET28a and *TM-NCGΔ*-pET28a, respectively.

Expression of gene and purification of gene product

E. coli BL21(DE3)CodonPlus-RIL strain (Invitrogen, Carlsbad, CA, USA) was transformed by the expression plasmid carrying the gene encoding SmDex95 or the five TMs. Each transformant was grown in Luria-Bertani medium (Becton and Dickinson; 1,500 ml for SmDex95; 200 ml for TM-NCGΔ and TM-NCGΔ; 40 ml for TM-NCΔΔ, TM-ΔCΔΔ, and TM-ΔCsΔΔ) containing 30 μg/ml of kanamycin at 37°C until reaching an absorbance of 0.5 at 600 nm. Each culture was then agitated (170 rpm) for 30 min at 18°C and treated with 0.2 mM isopropyl β-D-thiogalactopyranoside overnight at 18°C. Cells were harvested by centrifugation at 8,000 x *g* for 20 min at 4°C, and suspended in 20 mM sodium phosphate buffer (pH 6.8) containing 0.5 M NaCl (buffer A) and 1.0 mM PMSF. The bacteria were disrupted by sonication followed centrifugation at 14,000 x *g* for 20 min at 4°C. The supernatant having enzyme activity (65 ml for SmDex95; 11 ml for TM-NCGΔ and TM-ΔCGΔ) was loaded onto a Ni-chelating Sepharose Fast Flow (Amersham Pharmacia) column (1.4 x 10.4 cm for SmDex95; 1.5 x 1.7 cm for TM-NCGΔ and TM-ΔCGΔ) equilibrated with buffer A. The column was washed with buffer A containing 20 mM imidazole, followed by elution with a linear gradient of 20-300 mM imidazole in buffer A. The fraction containing the purified TM was dialyzed against 20 mM potassium phosphate buffer (pH 6.6; buffer B) and concentrated by ultrafiltration using an Amicon Ultra 10,000 MWCO (Millipore, Billerica, MA, USA). Sodium azide (final concentration 2

mM) was added to the purified TM to prevent the growth of contaminating microorganisms during storage at 4°C.

For further purification of SmDex95 (due to coexistence of SmDex90-like protein), ammonium sulfate (final concentration 1 M) was added to the active fractions obtained from the Ni-chelating Sepharose chromatography. The sample was loaded onto a Butyl-TOYOPEARL 650M column (2.0 x 10 cm; TOSOH, Tokyo, Japan) equilibrated with buffer B containing 1.0 M ammonium sulfate. After washing the column with the same buffer, the absorbed proteins were eluted with a linear gradient (1–0 M) of ammonium sulfate in buffer B. The active fraction absorbed to the hydrophobic resin was dialyzed against buffer B, subjected to chromatography using a DEAE-TOYOPEAL 650M column (1.5 x 6.3 cm; TOSOH), washed with buffer B, and eluted with a linear gradient of 0–1.0 M NaCl in buffer B. The active fractions were dialyzed against buffer B, concentrated using a CentriPrep YM-30 (Millipore), mixed with 2 mM NaN_3, and stored at 4°C. All purification steps were conducted at 4°C.

Protein and enzyme assays

Protein assay for crude extract was performed by the Bradford's method (Bradford 1976) with bovine serum albumin (Sigma-Aldrich) as a standard. Concentration of the purified enzyme was calculated from the amino acid contents of its hydrolysate (6 N HCl for 24 h at 110°C; 25 μg sample) using an Amino Tac JLC-500/V amino acid analyzer (JEOL, Tokyo, Japan). Dextranolytic activity was measured by incubating a reaction mixture consisting of

0.4% dextran T2000, 40 mM sodium acetate buffer (pH 5.0 for SmDex90 and Smdex95; pH 5.2 for TM-NCGΔ and TM-ΔCGΔ) and an appropriate amount of enzyme at 35°C for 10 min. The amount of the produced reducing sugar was determined by the copper bicinchoninate method (McFeeters 1980) using isomaltose as the standard sugar. One unit of the enzyme activity was defined as the quantity of enzyme generating 1 μmol of reducing sugar per min under the above conditions.

The optimum pH of dextranolytic activity was evaluated by incubating a reaction mixture containing 140 mM of Britton-Robinson buffer (pH 3.0–8.0), 0.4% dextran T2000, and enzyme (SmDex90, 3.87 ng μl^{-1}; TM-NCGΔ, 0.947 ng μl^{-1}; TM-ΔCGΔ, 0.902 ng μl^{-1}) at 35°C. For pH-stability, enzymes (SmDex90, 38.7 ng μl^{-1}; TM-NCGΔ, 9.47 ng μl^{-1}; TM-ΔCGΔ, 9.02 ng μl^{-1}) were maintained for 20 h at 4°C in 140 mM Britton-Robinson buffer (pH 2.5–12.0) and the remaining dextranolytic activity was measured by reacting the treated enzyme with 0.4% dextran T2000 containing 140 mM sodium acetate buffer (pH 5.0 for SmDex90; pH 5.2 for TM-NCGΔ and TM-ΔCGΔ) at 35°C. For thermal stability, enzyme (SmDex90, 20.5 ng μl^{-1}; TM-NCGΔ, 6.31 ng μl^{-1}; TM-ΔCGΔ, 3.01 ng μl^{-1}) was treated at 20–65°C for 15 min in 20 mM MES-NaOH buffer (pH 6.4, SmDex90) or 20 mM sodium phosphate buffer (pH 6.6 for TM-NCGΔ or TM-ΔCGΔ), and the residual activity at the optimum pH in 40 mM sodium acetate buffer was determined.

For determining substrate specificity, the initial velocity was measured by reducing power generation from substrate using 40 mM sodium acetate buffer (optimum pH for each enzyme) and 0.564–155 ng μl^{-1} of enzyme. The kinetic parameters, k_{cat} and K_m, for hydrolytic reaction on dextran T2000 (0.05-1.0%) using enzyme (SmDex90, 1.94 ng μl^{-1};

TM-NCGΔ, 1.26 ng μl^{-1}; TM-ΔCGΔ, 0.752 ng μl^{-1}) were estimated by fitting the initial velocities to the Michaelis-Menten equation using the CurveExpert 1.3 software (http://www.curveexpert.net/).

Gel electrophoresis and protein analysis

The purity of the enzyme was confirmed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Mark 12 molecular mass standard (25–200 kDa; Invitrogen), followed by staining with Coomassie Brilliant Blue (Laemmli 1970). Activity staining was conducted using 10% SDS-PAGE in the presence of 0.5% blue dextran 2000 (Barrett et al. 1987). Dextranolytic activity was detected by the formation of a clear band on a blue background of blue dextran, after the protein in gel was renatured by incubation for 1 h with 20 mM sodium phosphate buffer (pH 6.6) containing 0.5% (v/v) Triton X-100 which removed SDS from gel. Amino-acid sequence was analyzed with a Procise model 490 automated protein sequencer (Applied Biosystems). After the electrophoretically resolved protein was electrically transferred to a ProSorb membrane (Applied Biosystems), a piece of membrane carrying protein was subjected to the protein sequencing. The molecular mass of the protein was measured by SDS-PAGE, electrospray ionization-mass spectrometry (ESI-MS) using a JMS-700TZ apparatus (JEOL), and the aforementioned amino acid composition analysis.

Sequence alignment and prediction of signal sequence

Similarity searches were conducted at the Swiss Institute of Bioinformatics using the BLAST network service (http://www.isb-sib.ch). The BLAST 2 searches (Altschul et al. 1997) were performed using the Swiss-Prot/TrEMBL database. Signal peptide sequence was predicted using the Signal P (http://www.cbs.dtu.dk/services/SignalP/) server (Nielsen et al. 1997).

Results

Cloning of SmDex gene

A DNA fragment harboring SmDex gene (2,553 bp) was amplified by two independent PCR procedures and sequenced (GenBank accession, HQ711852). The DNA sequences were completely identical, confirming no PCR error. The deduced amino-acid sequence of SmDex (composed of 850 residues with a molecular mass of 94,537 Da; Fig. 1) showed an extremely high identity with that of Dex from *S. mutans* Ingbritt (850 residues; accession ID: AAN59642; Igarashi et al. 1995b). Nine amino acids of Ala16, Thr22, Val39, Gly56, Ser67, Ser766, Arg802, Ser805, and Lys849 in Ingbritt Dex were Asn, Ala, Ala, Glu, Pro, Asn, Gln, Pro, and Asn in our SmDex, respectively. All replacements were situated at N-VR or C-VR. The schematic structure of SmDex is shown in Fig. 2. Two acidic amino acids (Asp258 and Asp385) in CR respectively corresponded to the two catalytically essential amino acids, Asp145 and Asp270, of CITase (Yamamoto et al. 2006).

Expression and isolation of SmDex95, and characterization of SmDex90

Table 1 summarizes the purification procedure of SmDex 90 and SmDex95. Recombinant SmDex95, which harbored His6-tags at the N- and C-termini, was generated using *E. coli*. Cell-free extract (1,060 U with 0.85 U mg^{-1}) was subjected to chromatography using Ni-chelating resin. Dextranolytic activity was eluted at the resin-absorbed fractions.

Activity staining using SDS-PAGE revealed two active components with different molecular masses of 95 kDa (SmDex95) and 90 kDa (SmDex90). SmDex95 adsorbed to the resin more strongly than did SmDex90, since it was eluted by higher concentration of imidazole. A chromatography procedure using hydrophobic Butyl-TOYOPEARL separated SmDex95 and SmDex90 as the absorbed and non-absorbed fractions, respectively. SmDex90 migrated as a single band on SDS-PAGE (Fig. 3A, lane 2) and displayed dextranolytic activity (Fig. 3B, lane 2). SmDex90 was obtained with a yield of 41.4 mg and a specific activity of 8.21 U mg^{-1}. Hydrophobic resin-absorbed fractions containing SmDex95 retained an extra protein of SmDex90-like enzyme. A further round of chromatography using DEAE-TOYOPEAL failed to produce a single band of SmDex95 (lane 1 in Figs. 3A and 3B).

Purified SmDex90 endowisely hydrolyzed dextran T2000 to produce IG2 and IG3 as final products. Its optimum pH was at pH 5.0. The pH- and temperature-stability ranges (maintaining more than 95% of original activity) were at pH 5.0–8.8 and <40°C, respectively. Substrate specificity is mentioned subsequently.

Analysis of cleavage site of digested SmDex

Six months after purification of SmDex90, we found many protein bands upon SDS-PAGE (lane 3 in Fig. 3A), although the specific activity was not changed. This degradation was also observed in preparation of SmDex95 (DEAE-TOYOPEARL-absorbed fraction). During 6 months of storage, both SmDex90 and SmDex95 were stored at 4°C with 2 mM

NaN_3. No growth of microbial contaminants occurred, as evaluated by microscope observation. Incubation of newly-purified SmDex90 with 0.1 mM PMSF reduced this proteolytic degradation, implying the presence of a slight amount of Ser-protease. Activity staining (lane 3 in Fig. 3B) indicated that the generated small polypeptide with 69.7 kDa (band c; SmDex70) or 58.1 kDa (band d; SmDex58) displayed dextranolytic activity, with the remaining five polypeptides that were <55.5 kDa (bands e–i) displaying no activity.

N-Terminal amino-acid sequences of eight digested polypeptides (bands b to i in Fig. 3A) and SmDex95 (band a) were analyzed to determine their protease-cleavage sites. Table 2 summarizes the N-terminal amino-acid sequences and the putative C-terminal amino acids, the latter of which were estimated by the molecular mass using SDS-PAGE, ESI-MS, and amino acid contents. The N-terminal amino-acid sequences of SmDex95 and SmDex90 were GSSHH, originating from the N-terminal His6-tag. SmDex95 was thought to contain full-length original SmDex protein with double His6-tags at the N- and C-termini (Fig. 2). ESI-MS analysis determined the molecular mass of SmDex90 to be 89,835 kDa, indicating that SmDex90 was composed of Ser25-Ser807 with an N-terminal His6-tag (Fig. 2). The catalytically active SmDex58 and SmDex70 started with Gln100 and Ser60 and had a putative C-terminus at Tyr732 and Phe604, respectively (Table 2). Two polypeptides maintained the enzyme activity, even while lacking the N-VR and C-VR (SmDex70) or the GBS/C-VR (SmDex58) (Fig. 2). Five other catalytically inactive polypeptides (bands e-i) did not harbor full-size CR, the missing region of which contained the essential Asp258 or/and Asp385 (Fig. 2). To evaluate the functions of the degraded regions, particularly of N-VR, GBD and C-VR, five TMs were constructed and characterized.

Characterization of TMs

Figure 2 shows the schematic structure of five TMs constructed by the truncation approach. All TMs were generated using *E. coli*, but TM-NCΔΔ, TM-ΔCΔΔ and TM-ΔCsΔΔ were not expressed. These three TMs were devoid of GBS, suggesting that the GBS gene is important for SmDex expression. TM-NCGΔ and TM-ΔCGΔ were purified by the one-step chromatography with Ni-chelating resin (Fig. 3C); the yields (specific activities) of TM-NCGΔ and TM-ΔCGΔ were 12.3 mg (9.71 U mg^{-1}) and 9.47 mg (23.4 U mg^{-1}), respectively.

The optimum pH of TM-NCGΔ and TM-ΔCGΔ (pH 5.2) was almost identical to that of SmDex90 (pH 5.0). Deletion of N-VR and C-VR did not affect the thermal stability (<40°C). The pH-stability ranges were pH 5.2–8.8 for TM-NCGΔ and pH 5.2–8.2 for TM-ΔCGΔ, the latter of which slightly narrowed the range at alkaline side by pH 0.6 unit (pH 5.2–8.8 for SmDex90).

The proteolytic digestion of TM-NCGΔ and TM-ΔCGΔ was assessed after a prolonged storage of 2 months at 4°C (Figs. 3D and 3E). No TM-ΔCGΔ proteolytic degradation was observed. However, TM-NCGΔ was degraded to produce an active 72.5 kDa polypeptide with the similar molecular mass to TM-ΔCGΔ, suggesting that its N-VR was digested with a slight amount of coexisting protease. These results indicated that TM-ΔCGΔ is a robust enzyme that is resistant to protease-digestion.

Substrate specificities and kinetic parameters

Substrate specificities of SmDex90, TM-NCGΔ and TM-ΔCGΔ were assessed by measuring the initial velocities toward a variety of substrates (Table 3). TM-NCGΔ exhibited almost a similar hydrolytic activity on 0.4% dextran T2000 to that of SmDex90, and increased the activity by 1.4-fold or 2.0-fold at 0.05% dextran T2000 or T10, respectively, and by 1.6- to 2.4-fold at small substrates of CI-8, pNP-IG3 and pNP-IG4. TM-ΔCGΔ enhanced the activity on all substrates tested by 1.8- to 7.2-fold. Kinetic parameters of the enzymes on dextran T2000 were estimated (Table 4). The protein size-dependent reduction of K_m value was observed at TM-NCGΔ and TM-ΔCGΔ, while the k_{cat} values were not altered by the truncation. The k_{cat}/K_m value increased mainly due to the decrease of K_m value.

Discussion

Multiple isoform formation

Two types of mechanisms have been proposed on the multiple-form formation of GH 66 Dexs: i) multiple genes for Dexs (isozymes) (Finnegan et al. 2004a; Oguma et al. 1999) and ii) proteolytic degradation (isoforms) (Igarashi et al. 1995a, 2001; Lawman and Bleiseis 1991). In the case of GH 66 Dexs, however, the main reason seems to be post-translational modification of proteolytic degradation, since there were many examples reported, including this study. GH 66 Dexs have the different lengths of N-VR and C-VR with low amino-acid sequence similarity (Fig. 1). Those regions might be sensitive to protease attack (Khalikova et al. 2003). In particular, *Streptococcus* Dexs harbor longer N-VR and C-VR than any other enzyme [e.g. Dex from *Paenibacillus* sp. (Finnegan et al. 2004a); Fig. 1].

One possible function of the long C-VR of SmDex is the binding to cell wall of gram-positive bacteria, since V-CR harbors a cell wall-anchoring region, including the sorting signal of LPXTG motif (811-LPQTG-815, Fig. 1) (Paterson and Mitchell 2004). Thr of this motif may covalently link to the peptidoglycan by transpeptidase (sortase) together with the cleavage of Thr-Gly. Following this sorting motif was a charged heptapeptide (817-NNETRSN-823) and then 21 highly hydrophobic amino acids (824-LLKVIGAGALLIGAAGLLSLI-844), possibly staying in bacterial cytoplasmic membrane. SmDex terminated with a positively charged hexapeptide (845-KGRKND-850), reaching the inside of cell. These regions were structurally similar to the cell

wall-anchoring region (Igarashi et al. 2004b). However, the effect of a long C-VR, as well as of long N-VR, on the catalytic function of SmDex remains to be clearly elucidated.

In this study, SmDex95 were degraded by protease(s) of *E. coli* during its gene expression and long storage. The first degradation occurred at Gln808-Ala809 of C-VR, situated just prior to the aforementioned cell wall sorting signal. This digestion produced an isoform of SmDex90 with a single His6-tag at the N-terminus, which was eluted by lowered imidazole concentration in Ni-chelating chromatography than was SmDex95, which possessed double His6-tags. There is a possibility that SmDex95 was gradually digested to form SmDex90 during purification procedure even after Ni-chelating chromatography, resulting in the co-existence of SmDex90-like protein in SmDex95 preparation (lane 1 of Figs. 3A and 3B). Surprisingly, we found further proteolytic degradation of purified SmDex90 (lane 3 in Fig. 3A) during 6 month-storage at 4°C with 2 mM NaN_3. Chymotrypsin and Glu-C protease are candidates to attack the peptide bonds of V59-S60 and E557-T558, respectively (Table 2). The cleavage at Asn99-Gln100, Ser283-Gln284 and Ser807-Gln808 may be accomplished i) by broad specificity protease, which is similar to subtilisin or proteinase K, and/or ii) by an as-yet unidentified protease of "Gln-N protease". To our knowledge, there has been no report of a "Gln-N protease", which specifically cleaves the peptide bond of X-Gln.

Truncation of C-VR and N-VR

Enzymatically active component of SmDex70 was found during prolonged storage of

SmDex90 (lane 3, Fig. 3B). SmDex70 was devoid of the intact N-VR and C-VR, suggesting that both N-VR and C-VR are not crucial for dextranolytic activity. The present truncation studies also support this suggestion, since TM-NCGΔ and TM-ΔCGΔ were active enzymes. Therefore, neither N-VR nor C-VR is essential for the catalytic activity of GH 66 Dex. The result obtained with TM-NCGΔ agrees with the finding by Morisaki et al. (2002) that the mutant Dex from *S. mutans* Ingbritt, carrying an 83%-truncated C-VR, still maintained the dextranolytic activity. But an Ingbritt Dex carrying further truncation of N-terminal 111 amino acids (a mutant having double deletion of 83% C-VR and 61% N-VR) did not exhibit any enzyme activity (Morisaki et al. 2002), which does not agree with the present results for TM-ΔCGΔ. In the study of Morisaki et al. (2002), an activity staining with blue dextran-containing SDS-PAGE was the only way to detect dextranolytic activity, indicating the necessity of the refolded protein structure. Probably, the remaining 39% portion of the N-VR inhibited the renaturation of this double-truncated protein. Interestingly, the GBS/C-VR-deficient SmDex58 (Fig. 2) retained enzyme activity (band d, lane 3, Fig. 3B). SmDex58 is putatively composed of only CR and defective N-VR, suggesting that GBS is not also essential. We could not demonstrate this suggestion, since our attempt to generate TM-NCΔΔ, TM-ΔCΔΔ, and TM-ΔCsΔΔ was unsuccessful. A DNA region for GBS may be necessary for the expression of those TMs. The smallest SmDex currently available is a TM-ΔCGΔ harboring CR and GBS.

Prolonged storage (6 months) converted the SmDex90 to a main active component of SmDex70 and many catalytically inactive polypeptides (lane 3 in Fig. 3B) without the loss of specific activity. Therefore, SmDex70 lacking both N-VR and a portion of C-VR (Fig. 2)

was considered to have greater enzyme activity than SmDex90. From these findings, we expected that N-VR and C-VR might decrease the enzyme activity of SmDex. Our expectation was supported by TM-NCGΔ and TM-ΔCGΔ, both of which were found to enhance their activities to all substrates tested (Table 3). Their affinities to dextran T2000, evaluated by K_m values in Table 4, were higher than that of SmDex90 by 1.7-fold for TM-NCGΔ and by 0.58-fold for TM-ΔCGΔ. Although we have no reasonable explanation for this alternation, one possibility is that the catalytic site of SmDex90 is covered by N-VR and C-VR (Fig. 4A). By deletion of N-VR and C-VR (Fig. 4B), the catalytic site may have been exposed to allow easier approach of substrate molecule to the site, thus resulting in enhancement of their own activities. Both N-VR and C-VR are not considered to influence the sugar-binding function of GBS, since the activities to small substrate molecules (pNP-IG3, pNP-IG4, and CI-8) were also enhanced by deletion. Further studies of function of two VRs will require three-dimensional determination.

Why do most of GH 66 Dexs have the protease-scissile N-VR and C-VR? The Dex bearing two VRs is considered to be a proenzyme, which is activated by proteolytic digestion at both of N-VR and C-VR (Fig. 4C). The activation mechanism is similar to those of trypsinogen and chymotrypsinogen, both of which are also activated by digestion of their polypeptide chains. Generally, those proenzymes display slight activity. In the case of SmDex, the proenzyme is SmDex95. Its specific activity is also considered to be much smaller than 3.02 U mg^{-1} (Table 1), since the unpurified SmDex95 fraction (DEAE-TOYOPEARL-absorbed fraction having 3.02 U mg^{-1}) contained original SmDex95 and SmDex90-like component (lane 1 of Fig. 1A), the latter of which possibly has high

activity (8.21 U mg^{-1} of SmDex90). Purification and characterization of original SmDex from *S. mutans* ATCC 25175 are currently in progress.

There are several advantages of our SmDex TMs: i) both TM-ΔCGΔ and TM-NCGΔ enhance their enzyme activities; ii) TM-ΔCGΔ does not accept any further protease attack after expression in *E. coli*; iii) deletion of C-VR or N-VR/C-VR is not associated with an optimum pH, thermal stability, and pH-stability, except for only the reduced pH-stability of TM-ΔCGΔ at alkaline region by 0.6 pH unit. Our truncation approach may contribute to the industrial application of GH 66 Dex.

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Table 1 Isolation of SmDex95 and SmDex90

Purification procedure	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Recovery (%)
Crude extract	1,060	1,250[a)]	0.85	100
Ni-Chelating Sepharose	566	117[b)]	4.85	53.4
BUTYL-TOYOPEARL				
non-absorbed fraction (SmDex90)	340	41.4[c)]	8.21	32.1
absorbed fraction (SmDex95)	28.6	9.88[b)]	2.89	2.70
DEAE-TOYOPEARL (SmDex95)	25.4	8.40[b)]	3.02	2.40

[a)] measured by the Bradford's method (Bradford 1976)

[b)] calculated from A_{280} on the assumption of $E^{1\%}_{1cm} = 10.0$

[c)] calculated from A_{280} based on $E^{1\%}_{1cm} = 8.66$

Table 2 N-Terminal sequence and predicted C-terminus of polypeptide

Band[a)]	Activity[b)]	Molecular mass (kDa)[c)]	N-terminal sequence[d)]	Cleavage site in N-terminal[d, e)]	Predicted C-terminal[d, e, f)]
a	+	95.4	GSSHH[g)]	No digestion	C-End His6
b	+	89.8	GSSHH[g)]	No digestion	S807↓Q808
c	+	69.7	100-QKNGN	N99↓Q100	Y731↓I732
d	+	58.1	60-SANTN	V59↓S60	F604↓T605
e	–	55.5	284-QVETD	S283↓Q284	E791↓N792
f	–	32.9	284-QVETD	S283↓Q284	F582↓I583
g	–	30.3	284-QVETD	S283↓Q284	Y560↓Y561
h	–	27.5	558-TAYYP	E557↓T558	S807↓Q808
i	–	23.0	100-QKNGN	N99↓Q100	S283↓Q284

[a)] Polypeptide band shown in Fig. 3A

[b)] Determined by activity staining in Fig. 3B

[c)] Calculated by SDS-PAGE (Fig. 3A) for all polypeptides, and by ESI-MS and amino acid composition for SmDex90 (band b)

[d)] Number showing the position of the amino-acid sequence of SmDex (Fig. 1)

[e)] Arrow showing the proteolytically digested site

[f)] Presumed from the analyzed N-terminal sequence and molecular mass

[g)] N-Terminal His6-tag sequence

Table 3 Substrate specificities of SmDex90, TM-NCGΔ, and TM-ΔCGΔ

Substrate	SmDex90	TM-NCGΔ	TM-ΔCGΔ
	v[a)] (Rel. v)[b)]	v[a)] (Rel. v)[b)]	v[a)] (Rel. v)[b)]
0.4% Dextran T2000	12.3 (100)	13.4 (109)	28.3 (230)
0.05% Dextran T2000	2.15 (100)	2.91 (135)	10.1 (470)
0.05% Dextran T10	4.52 (100)	9.09 (201)	32.5 (719)
10 mM CI-8	0.464 (100)	1.13 (244)	1.38 (297)
4 mM pNP-IG3	0.00425 (100)	0.00682 (160)	0.00907 (213)
2 mM pNP-IG4	0.0294 (100)	0.0482 (164)	0.0531 (181)

Deviation <5% for each value

[a)] μmol product/sec/μmol enzyme

[b)] Relative v (%)

Table 4 Kinetic parameters for dextran T2000

Enzyme	K_m (%)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot \%^{-1}$)
SmDex90	0.840	38.3	45.6
TM-NCGΔ	0.502	31.9	63.5
TM-ΔCGΔ	0.145	38.3	264

Deviation <5% for each value

Figure Legends

Fig. 1 Amino-acid sequence alignment of GH 66 enzymes.

The sequence alignment was done using National Center for Biotechnology Information Basic Local Alignment Search Tool (Altschul et al. 1997). SRDex, Dex from *S. ratti* ATCC 19645 (GeneBank ID: BAD12421); SMDex, Dex from *S. mutans* ATCC 25175 (HQ711852); SMDexUA, Dex from *S. mutans* UA159 (AAN59642); PspDex, Dex from *Paenibacillus* sp. (AAQ91301); BciTCIT, CITase from *B. circulans* T-3040 (BAA09604). The two deduced catalytic Asps of GH 66 enzymes are enclosed with a square. Portions with a green underline and a blue underline are the signal sequence and CITase-specific region, respectively. Signal P server predicted the signal peptides of SRDex, SMUDex, and PspDex.

Fig. 2 Deletion mutant of SmDex.

Upper numbers correspond to amino acids of full-size SmDex. , signal peptide; , N-VR; , CR; , GBS; , C-VR; and , catalytically important Asp258 and Asp385, respectively; **H**, His6-tag; +, having enzyme activity; -, not having enzyme activity; NE, not expressed. Molecular masses were estimated by SDS-PAGE (for all cases), EIS-MS (for SmDex90), and amino acid composition analysis (for SmDex90, TM-NCGΔ, and TM-ΔCGΔ).

Fig. 3 SDS-PAGE of SmDex.

Molecular masses of standard proteins are displayed on the left. Protein bands

numbered from a–i were used for the analysis of N-terminal amino-acid sequence (Table 2). (A), (C) and (D) depict results of protein staining. (B) and (E) depict results of activity staining. Lane M, molecular size makers; lane 1, SmDex95 after DEAE-Sepharose column chromatography (Table 1); lane 2, purified SmDex90; lane 3, SmDex90 maintained for 6 months at 4°C after purification; lane 4, TM-NCGΔ; lane 5, TM-ΔCGΔ; lane 6, TM-NCGΔ stored at 4ºC for 2 months; lane 7, TM-ΔCGΔ stored at 4ºC for 2 months.

Fig. 4 Predicted structures of SmDex90, SmDex95, TM-NCGΔ, and TM-ΔCGΔ.

(A) Proteolytic digestion of SmDex95 to form SmDex90. (B) Structures of TM-NCGΔ and TM-ΔCGΔ. (C), Conversion of less active SmDex (proenzyme) to activated form by degradation of N-VR and/or C-VR. Black triangle on CR is a catalytic site. In (A), the C-terminal 42 amino acids of C-VR are deleted by proteolytic digestion.

Fig. 1

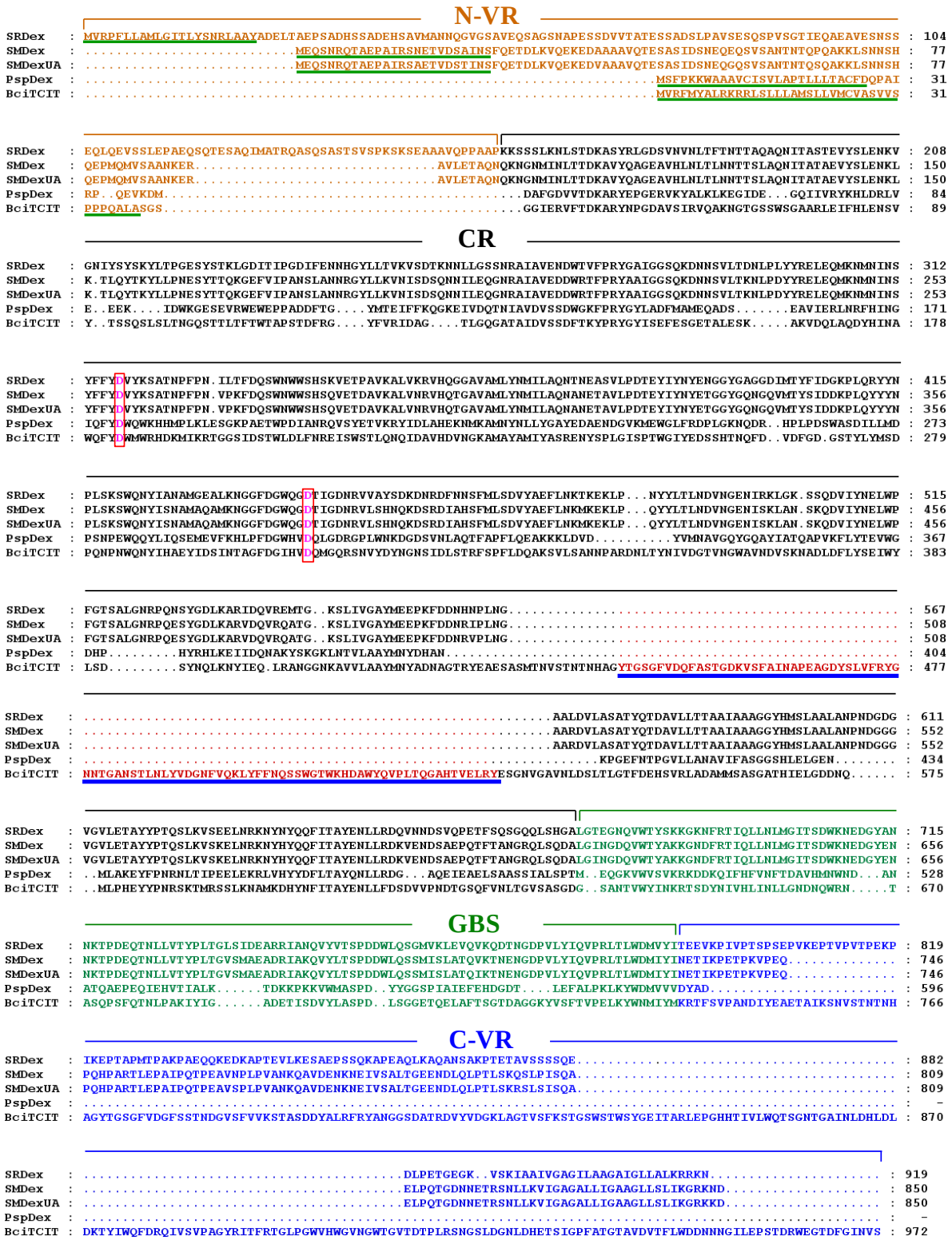


Fig. 2

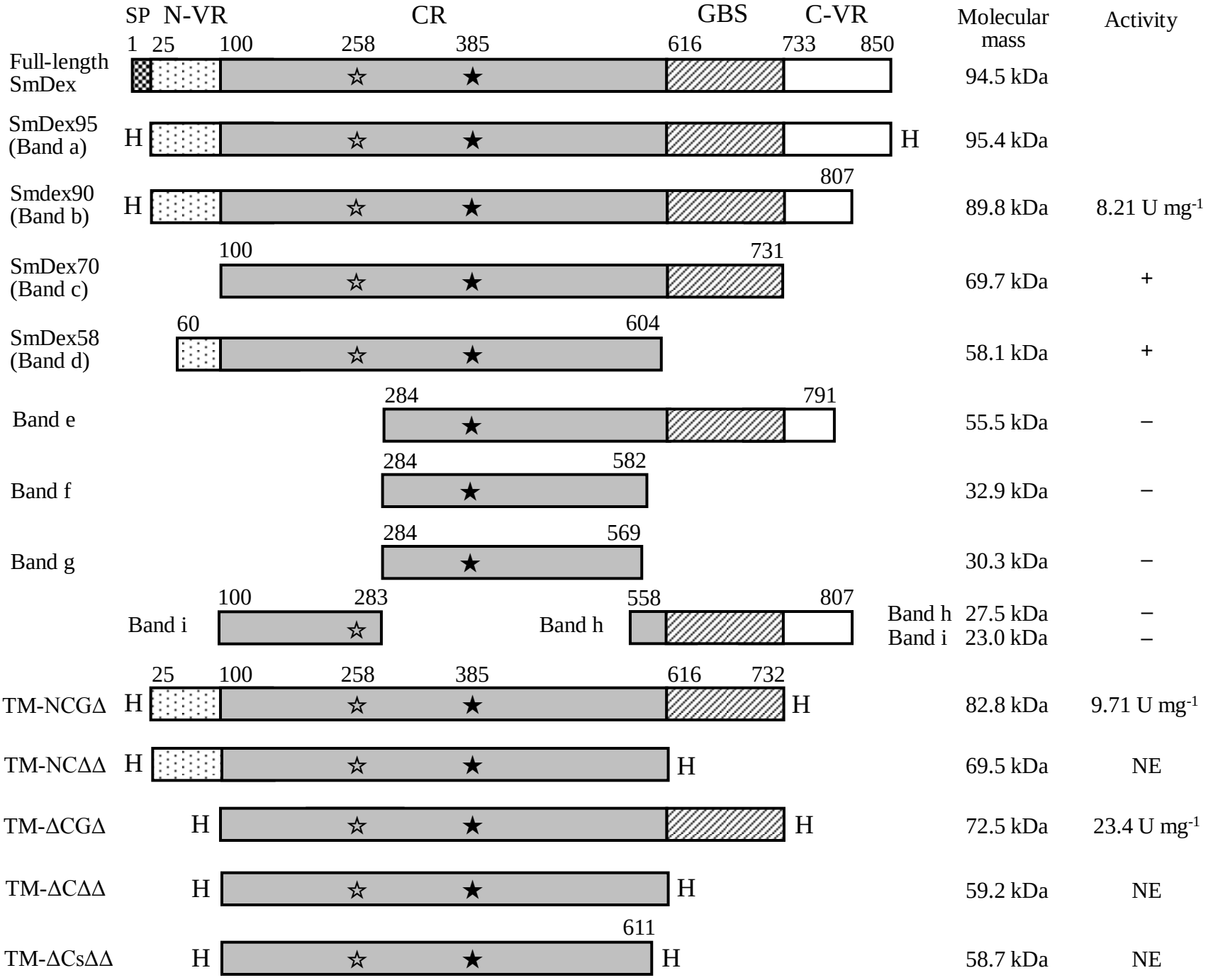

SP
N-VR
CR
GBS
C-VR
Molecular mass
Activity
1
25
100
258
385
616
733
850
Full-length SmDex
94.5 kDa
SmDex95 (Band a)
H
H
95.4 kDa
807
Smdex90 (Band b)
89.8 kDa
8.21 U mg-1
731
SmDex70 (Band c)
69.7 kDa
+
60
604
SmDex58 (Band d)
58.1 kDa
+
284
791
Band e
55.5 kDa
−
582
Band f
32.9 kDa
−
569
Band g
30.3 kDa
−
283
558
Band i
Band h
Band h 27.5 kDa
Band i 23.0 kDa
−
−
732
TM-NCGΔ
82.8 kDa
9.71 U mg-1
TM-NCΔΔ
69.5 kDa
NE
TM-ΔCGΔ
72.5 kDa
23.4 U mg-1
TM-ΔCΔΔ
59.2 kDa
NE
611
TM-ΔCsΔΔ
58.7 kDa
NE

Fig. 3

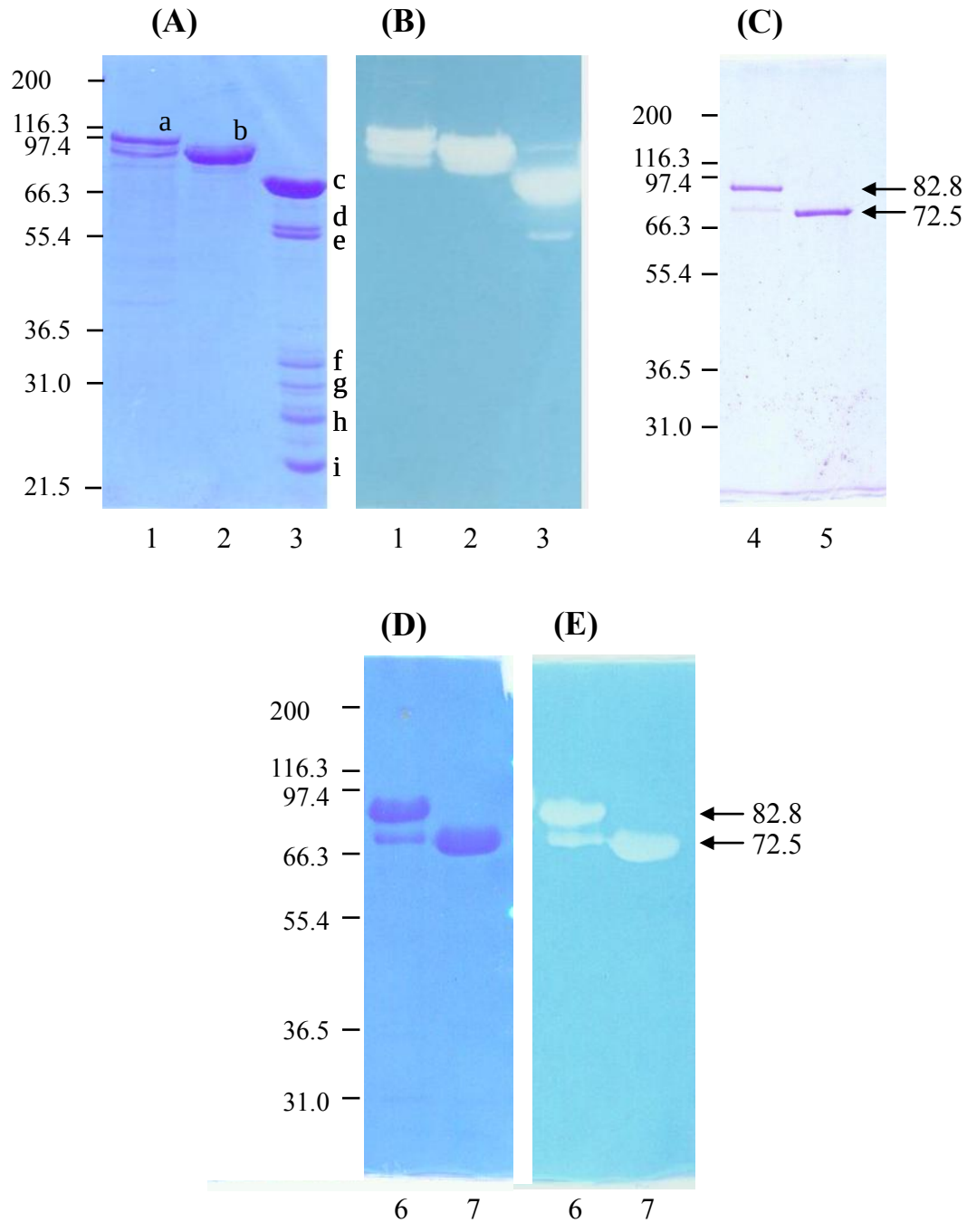
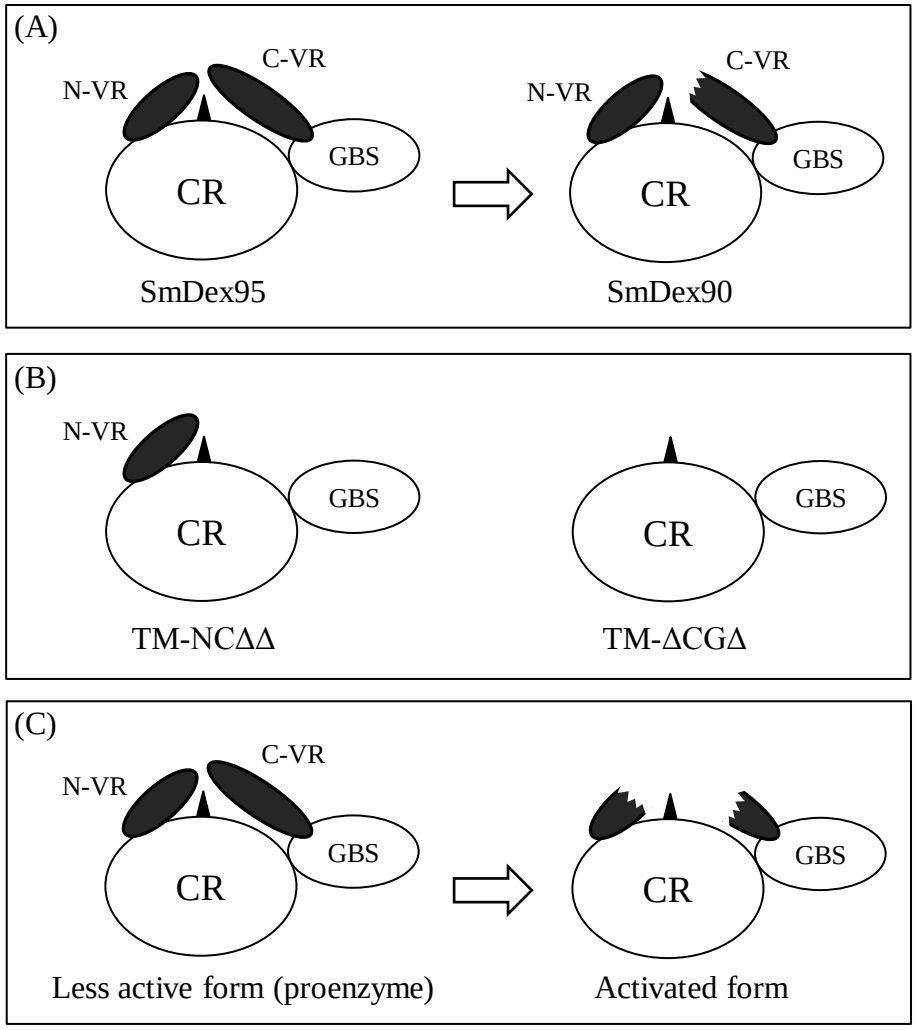


Fig. 3 (Kim et al.)

Fig. 4


(A)
C-VR
N-VR
GBS
CR
SmDex95
C-VR
N-VR
GBS
CR
SmDex90
(B)
N-VR
GBS
CR
TM-NCΔΔ
GBS
CR
TM-ΔCGΔ
(C)
C-VR
N-VR
GBS
CR
Less active form (proenzyme)
GBS
CR
Activated form