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Biochemical characterization of a β-*N*-acetylhexosaminidase from *Streptomyces alfalfae* and its application in the production of *N*-acetyl-D-glucosamine

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_N_-Acetyl-D-glucosamine (GlcNAc) is a valuable monosaccharide widely used in the medical, agricultural, biofuel, and food industries. Its efficient and environment-friendly production depends on the binary system of β-_N_-acetylhexosaminidase (HEX) and chitinase. In the present study, a HEX of glycoside hydrolasefamily 20 was identified in _Streptomyces alfalfae_ ACCC40021, and was overexpressed in _Escherichia coli_. The purified recombinant _Sa_HEX showed maximal activities at 60°C and pH 5.5, and retained stable up to 45°C. The enzyme not only exhibited broad substrate specificity including _p_-nitrophenyl β-_N_-acetylglucosaminide, _p_-nitrophenyl β-_N_-acetylgalactosaminide, chitooligosaccharides and colloidal chitin, but also had higher specific activities (up to 1149.7 ± 72.6 U/mg) towards natural and synthetic substrates. When combined with a commercial chitinase, it achieved a conversion rate of 93.7% from 1% of colloidal chitin to GlcNAc in 6 h, with the product purity of >98%. These excellent properties make _Sa_HEX a potential enzyme candidate for the chitin conversion for various industrial purposes.

[**Key words:** *N*-Acetylhexosaminidase; *Streptomyces alfalfa*; *N*-Acetyl-D-glucosamine; Enzymatic conversion; Chitin]

N-Acetyl-D-glucosamine (GlcNAc) is a valuable monosaccharide widely applied in the medical, pharmacological, agricultural, biofuel, and cosmetic fields (1–4). In the food industry, it has attracted much attention due to the chemically stable characteristic and refreshing taste with anti-oxidation and immunity-enhancing activities (5,6). As the raw material of GlcNAc, chitin is a linear biopolymer of β-1,4-linked GlcNAc, and represents the second most abundant biomass on Earth with an annual synthesis rate of 10^{11} tons (7). Being the predominant component of crustacean exoskeletons, cuticle of insect and fungal cell walls, chitin can be converted into GlcNAc through chemical and enzymatic approaches. The enzymatic approach for chitin biodegradation requires the concerted action of two chitinolytic enzymes, i.e., endo-type chitinase (E.C. 3.2.1.14) that cleaves chitin into $(GlcNAc)_2$ and exo-type β-*N*-acetylhexosaminidase (HEX, E.C. 3.2.1.52) that further hydrolyzes $(GlcNAc)_2$ into GlcNAc (8). In comparison to the traditional harsh and heavily polluted chemical method, the enzymatic conversion strategy is superior in the advantages of environment-friendly, low cost and high efficiency (3,9).

HEXs are glycoside hydrolases that catalyze the cleavage of terminal GlcNAc and β-*N*-acetyl-D-galactosamine residues from the non-reducing end of *N*-acetyl-β-D-hexosaminides in oligosaccharides, glycoproteins, glycolipids, and glycosaminoglycans. They have attracted considerable attention due to the physiological importance in human neuro-degenerative diseases, recycling of cell walls in bacteria and fungi, and biocontrol of fungal and insect pests (10,11). Based on the amino acid sequence similarity, HEXs are classified into four glycosyl hydrolase (GH) families of 3, 20, 84, and 85 in the Carbohydrate-Active Enzyme Database (CAZy, http://cazy.org/). Of them, GH20 family contains the majority of HEXs, and has been deeply studied. With a dozen of crystal structures resolved, the catalytic mechanism of GH20 HEXs has been revealed: they commonly employ a substrate-assisted catalytic mechanism (12) and have been used in the biocontrol of phytopathogenic fungi, degradation of chitinous waste, and synthesis of new oligosaccharides drugs (8,13,14).

A large amount of GH20 HEXs from bacteria, fungi, insects, plants, and human have been identified, gene cloned, and characterized. However, some of them have low activities (15,16), or are sensitive to high temperature, which limit their commercial applications (17). Therefore, it is of great importance to obtain a novel HEX with high catalytic efficiency and stability for industrial applications. In the present study, the plant beneficial rhizobacterium, *Streptomyces alfalfae* ACCC40021, a commercial microbial fertilizer that can facilitate the conversion of carbon and nitrogen sources (18), was selected as the HEX gene donor. A β-*N*-acetylhexosaminidase, *Sa*HEX, was identified in strain ACCC40021 and successfully expressed in *Escherichia coli* BL21(DE3). The enzymatic properties of recombinant *Sa*HEX were determined, and its potential as a biocatalyst to convert chitin into GlcNAc was assessed.

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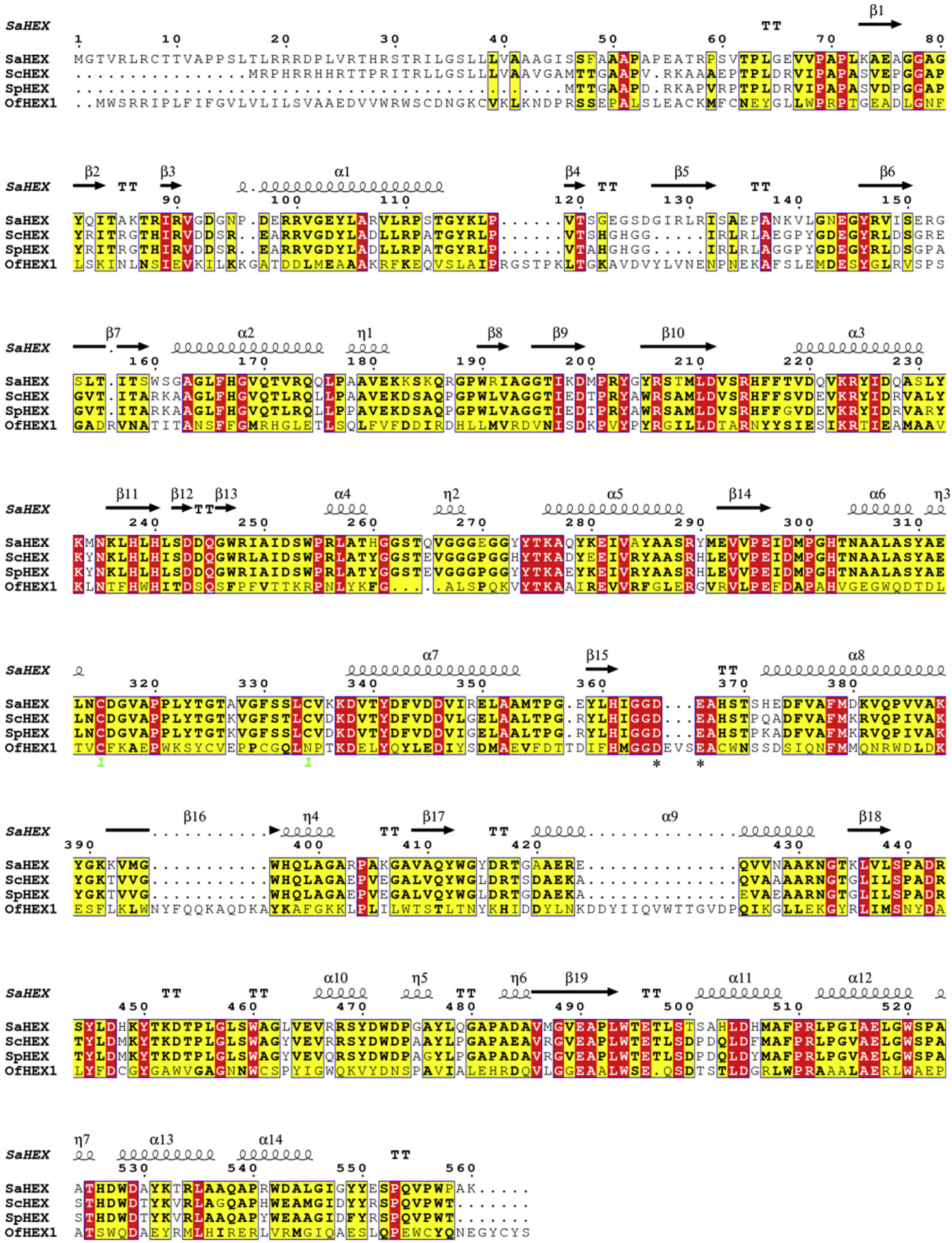
SaHEX
ScHEX
SpHEX
OfHEX1

MATERIALS AND METHODS

Strain and chemicals *S. alfalfae* ACCC40021 was obtained from the Agricultural Culture Collection of China (ACCC) and used as the donor strain of the *Sahex* gene. The plasmid pET-30a (+) (Invitrogen) and the host *E. coli* BL21(DE3) were used for heterologous expression. Restriction enzymes, T4 DNA ligase, and *Pfu* DNA polymerase were purchased from TransGen Biotech (Beijing, China). *p*-Nitrophenyl β-*N*-acetylglucosaminide (*p*NP-GlcNAc), *p*-nitrophenyl β-*N*-acetylgalactosaminide (*p*NP-GalNAc) and chitinase from *Streptomyces griseus* were purchased from Sigma–Aldrich. Chitooligosaccharides (CHOS) with the polymerization degree (DP) of 2–6 were provided by the BZ Oligo Biotech (Qingdao, China). All other chemicals were of analytical grade and commercially available.

Colloidal chitin was prepared from the chitin powder of shrimp shells as previously described (19) with some modifications. Briefly, 5 g of chitin powder were dissolved in 400 mL of concentrated HCl by gentle stirring for 4 h. Colloidal chitin was precipitated by adding 500 mL of 50% ethyl alcohol at 4°C with constant mixing for 24 h. The precipitate was collected by centrifugation at 10,000 × *g* for 10 min, washed with distilled water until the pH became neutral, and dissolved in 500 mL of distilled water to the final concentration of 1% (w/v). The colloidal chitin was stored at 4°C before use.

Cloning and sequence analysis of the *Sahex* gene The gene fragment coding for the *Sa*HEX (locus tag: A7J05_RS24005) was amplified by PCR using the primers 5′-CGCGGATCCGCCGCCCCCGCCCCC-3′ and 5′-AGCAAGCTTC-TACTTGGCGGGCCAGG-3′ (the *Bam*HI and *Hind*III restriction sites underlined) according to the genome sequence of *S. alfalfae* ACCC40021 (Genbank accession No. CP015588.1). The PCR product was purified and digested with *Bam*HI and *Hind*III, and inserted into the plasmid pET-30a (+) using its own stop codon. The recombinant plasmid pET-30a-*Sahex* was then transformed into *E. coli* BL21(DE3) competent cells for heterologous expression. Transformants were screened on LB plate supplemented with kanamycin (50 mg/l). The positive transformants were confirmed by DNA sequencing (BGI, Shenzhen, China).

Nucleotide and amino acid sequence alignments were conducted using the BlastX and BlastP programs of NCBI (http://www.ncbi.nlm.nih.gov/BLAST), and the multiple sequence alignment was generated using the ClustalW software (http://www.clustal.org/). SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP) was used to predict the presence of the signal peptide. The modeled structure of *Sa*HEX was predicted using the Swiss-Model server (http://www.swissmodel.expasy.org/) with the GH20 HEX from *Streptomyces coelicolor* A3(2) (4C7G, 71% identity) as a template.

Expression and purification of the recombinant *Sa*HEX *E. coli* BL21(DE3) cells harboring the pET-30a-*Sahex* were grown at 37°C in LB medium containing kanamycin (50 mg/L) with vigorous agitation until the OD_{600} value reached 0.5. Isopropyl 1-thio-D-galactopyranoside (IPTG) was then added at the final concentration of 0.1 mM to induce the production of recombinant *Sa*HEX. After 16-h cultivation at 20°C, the cells were collected by centrifugation, and suspended in the equilibration buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8.0). Cells were disrupted by sonication, and centrifuged at 15,000 × *g* for 10 min at 4°C to remove insoluble cell debris. The supernatants were applied to an Ni^{2+}-NTA agarose column, and eluted with elution buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole and 0.1% Triton X-100, pH 8.0. The fractions showing HEX activities were pooled and concentrated. SDS-PAGE was carried out on a 12% separation gel (20). Protein concentration was measured by using the Lowry method with bovine serum albumin as the standard (21).

Enzyme activity assays The standard reaction system contained 0.1 mL of diluted enzyme solution and 0.9 mL of 5 mM *p*NP-GlcNAc in 50 mM acetate buffer (pH 5.5). After incubation at 60°C for 10 min, the reaction was terminated by the addition of 3 mL of 0.5 M NaOH. Enzyme activity was then determined by measuring the absorption of liberated *p*-nitrophenol at 405 nm. One unit of enzyme was defined as the amount of enzyme to release 1 μmol of *p*-nitrophenol per min under above conditions.

When using CHOS of DP 2–6 (2 mM) as the substrate, high performance liquid chromatography (HPLC; Agilent 1260 Series, Agilent Technologies, Santa Clara, CA, USA) in conjunction with an evaporative light-scattering detector (ELSD) was used to determine the amount of GlcNAc. The hydrolysis products were analyzed on a SHODEX Amino-P50 4E column (Showa Denko, Tokyo, Japan). One unit of enzyme was defined as the amount of enzyme to release 1 μmol of GlcNAc per minute.

The 3,5-dinitrosalicylic acid (DNS) method (22) was used to determine the enzyme activity with 1% (w/v) of colloidal chitin as the substrate. One unit (U) of enzyme activity was defined as the amount of enzyme that produces 1 μmol of reducing sugars per minute under the above conditions using GlcNAc as the standard.

Biochemical characterization of purified *Sa*HEX The temperature-activity profile of purified recombinant *Sa*HEX was determined in 50 mM acetate buffer (pH 5.5) at temperatures from 30°C to 75°C. The pH-activity profile of purified recombinant *Sa*HEX was determined at 60°C and pH 4.0 to 9.0. The buffers used were acetate buffer (pH 4.0–6.0, 50 mM), sodium phosphate buffer (pH 6.0–8.0, 50 mM), and Tris/HCl buffer (pH 8.0–9.0, 50 mM).

For thermo-stability assay, the purified enzyme was incubated at 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C and 65°C for 1 h, followed by the residual activity assay towards *p*NP-GlcNAc. The stability of the purified *Sa*HEX at pH 4.0 to pH 9.0 was determined after 1 h incubation in different buffers.

The effects of metal ions and chemical reagents on *Sa*HEX were investigated by measuring the enzyme activity in the presence of 1 mM of KCl, $CaCl_2$, NaCl, $MgCl_2$, $ZnCl_2$, $FeCl_2$, $FeCl_3$, $CuSO_4$, $HgCl_2$, $AgNO_3$, $CoCl_2$, $NiCl_2$, $MnCl_2$, EDTA and β-mercaptoethanol. The activities of non-treated enzyme were defined as 100%.

The substrate specificity of *Sa*HEX was studied under standard conditions (pH 5.5 and 60°C for 10 min) with 5 mM of *p*NP-GlcNAc or *p*NP-GalNAc, 2 mM of CHOS of DP 2–6 or 1% (w/v) of colloidal chitin as the substrate.

The kinetic parameters, K_m and k_{cat}, of purified recombinant *Sa*HEX were determined by a nonlinear regression fit to the Michaelis–Menten equation using GraphPad Prism 5. The reaction systems contained 2 μg/mL of *Sa*HEX and *p*NP-GlcNAc of 0.1–1 mM in 50 mM acetate buffer (pH 5.5). The catalytic efficiency, k_{cat}/K_m, was then calculated.

Analysis of the CHOS hydrolysis products The hydrolysis products of CHOS by the purified *Sa*HEX were determined by using the thin layer chromatography (TLC). CHOS of DP 2–6 (2 mM) and 2 μg/mL of purified enzyme in 50 mM acetate buffer (pH 5.5) were incubated at 45°C for 1 h. Samples were taken at different time points, inactivated by boiling for 5 min, and stored at 20°C until analysis. Aliquots (2 μL) were separated on a silica gel plate (TLC Silica gel 60, Merck, Darmstadt, Germany) in a solvent system containing *n*-butyl alcohol/methanol/25% ammonia solution/water at the ratio of 5:4:2:1 (v/v/v/v) as developing solution. The hydrolysis products were detected by staining the plate with aniline-diphenylamine reagent (4 mL of aniline, 4 g of diphenylamine, 200 mL of acetone, and 30 mL of 85% phosphoric acid), followed by incubation at 180°C for 3 min.

Synergistic action on chitin conversion The synergistic action of *Sa*HEX and the commercial chitinase *Sg*Ctn was determined with colloidal chitin as the substrate. Reaction mixtures containing 1% (w/v) of colloidal chitin and either *Sg*Ctn (2 mg/mL), *Sa*HEX (50 μg/mL) or both enzymes were incubated at 45°C in 50 mM acetate buffer (pH 5.5) for 1 h. The amounts of reducing sugars liberated were then measured by using the DNS method. The reaction systems containing heat-inactivated enzymes were used as controls. The synergy degree was calculated by the fold changes of the amount of reducing sugars released by the enzyme combination against the sum of reducing sugar amounts released by individual enzymes.

To assess the application potential of *Sa*HEX in enzymatic conversion of colloidal chitin into GlcNAc, reaction systems containing 50 μg/mL of *Sa*HEX, 2 mg/mL of *Sg*Ctn and 1% (w/v) of colloidal chitin were incubated in 50 mM acetate buffer (pH 5.5) at 45°C for 24 h. Aliquots were taken at different times, and the reactions were terminated by adding an equal amount of 70% acetonitrile. The samples were stored at 20°C before HPLC analysis. Aliquots (2 μL) were injected into the HPLC-ELSD. The mobile phase consisted of 70% acetonitrile and 30% MilliQ H_2O, and the flow rate was set to 1 mL/min. Calibration curves were then constructed for GlcNAc. The conversion rate of colloidal chitin to GlcNAc was calculated as described (23).

Statistical analysis All data were shown as the mean of at least three independent replicates with their standard deviations (mean ± SD). GraphPad Prism 5 was used for statistical analysis, and the treatments with a p value of less than 0.05 were considered to be statistically significant.

Nucleotide sequence accession number The nucleotide sequence data reported in this paper was deposited in GenBank under the accession number MH900378.

RESULTS AND DISCUSSION

Sequence and structure analysis of *Sa*HEX The deduced *Sa*HEX contains a polypeptide of 530 amino acid residues with a calculated isoelectric point (*p*I) of 7.87 and a molecular mass of 55,664 Da. A putative signal peptide was predicted with a cleavage site between residues 31 and 32 by using the SignalP 4.1. *Sa*HEX displayed the highest identities with GH20 homologs from *Streptomyces* spp., 71% and 69% with *Sc*HEX from *S. coelicolor* A3(2) and *Sp*HEX from *Streptomyce splicatus*, respectively, but was phylogenetically distant from the *Of*HEX1 from *Ostrinia furnacalis* (insect; 21% identity) (Fig. 1). Multiple sequence alignment and structural prediction revealed that *Sa*HEX is a typical HEX of GH20, with the

FIG. 1. Multiple alignment of the amino acid sequences of *Sa*HEX and other GH20 homologs. *Sc*HEX, the HEX from *Streptomyces coelicolor* A3(2) (NP_627016); *Sp*HEX, the HEX from *Streptomyces plicatus* (AAC38798); and *Of*HEX1, the HEX from *Ostrinia furnacalis* (ABI81756). The identical amino acids are marked in red, and the conserved amino acids are marked in yellow. Structural model shown on the top of alignment based on *Sc*HEX (PDB entry: 4C7G).

putative catalytic residues of D365 and E366 in the catalytic module. Modeled *Sa*HEX presents a highly conserved $(\beta/\alpha)_8$-barrel (TIM-barrel) architecture of GH20 HEX (Fig. S1).

Expression and purification of the recombinant *Sa*HEX The His_6 tag fused on the N-terminus of mature *Sa*HEX was introduced from the expression vector pET-30a (+). The recombinant enzyme was successfully expressed in *E. coli* BL21(DE3) in a soluble form. It was purified to apparent homogeneity by Ni^{2+}-NTA chromatography with a recovery yield of 54.2%. The specific activity of purified recombinant *Sa*HEX was determined to be 1149.7 U/mg towards *p*NP-GlcNAc (Table S1). The apparent molecular mass of purified *Sa*HEX was approximately 60 kDa (Fig. S2), which is in agreement to its predicted size plus the N-terminal His_6 tag.

Biochemical characterization of the purified recombinant *Sa*HEX Despite *S. alfalfae*ACCC40021 is a mesophilic bacterium, purified recombinant *Sa*HEX exhibited thermophilic and acidic properties, exhibiting maximal activity at 60°C and pH 5.5 (Fig. 2A, B). The optimal temperature of *Sa*HEX is much higher than that of other GH20 members (mostly optimal at 30–50°C) (14,15,24–26). However, its pH and temperature adaptability was relatively narrow, remaining more than 80% of the maximum activity at pH 5.0–6.0 and 60–65°C, respectively. In comparison to the most characterized HEXs from mesophilic organisms that lose activities at temperatures higher than the optimal growth temperature (30–40°C) (14,18,27–30), *Sa*HEX was thermo-stable up to 45°C, retaining 85.3% activity even at 50°C (Fig. 2C). For pH stability, the enzyme retained stable at pH 4.5–8.5, with more than 80% of activity after 1-h incubation (Fig. 2D). Thus, the thermophilic and thermostable properties of *Sa*HEX make it attractive for industries.

The purified recombinant *Sa*HEX was highly resistant to most metal ions and chemical reagents tested (Table 1), including Fe^{3+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, K^+, Na^+, Ca^{2+}, Cu^{2+}, chelating agent EDTA and reductant agent β-mercaptoethanol. However, the enzyme activity was partially or markedly inhibited by 1 mM of Fe^{2+}, Zn^{2+}, Ag^+ and Hg^{2+}.

TABLE 1. Effect of metal ions and chemical reagents on the purified *Sa*HEX.[a]

Chemicals	Relative activity (%)	Chemicals	Relative activity (%)
$FeCl_3$	129 ± 4	$CaCl_2$	92 ± 2
$MgCl_2$	121 ± 3	EDTA	91 ± 1
$MnCl_2$	111 ± 3	β-Mercaptoethanol	91 ± 1
$CoCl_2$	104 ± 7	$FeCl_2$	72 ± 0
$NiCl_2$	102 ± 1	$ZnCl_2$	56 ± 3
KCl	100 ± 3	$AgNO_3$	25 ± 5
NaCl	97 ± 0	$HgCl_2$	5 ± 0
$CuSO_4$	94 ± 1	Control	100 ± 2

[a] Values represent means ± SD (n = 3) relative to the untreated control samples.

Substrate specificity and catalytic activity Purified *Sa*HEX showed broad substrate specificity (Table 2). It not only exhibited activity against *p*NP-GlcNAc and *p*NP-GalNAc, but also showed activity towards CHOS of DP 2–6 and colloidal chitin. When defined the specific activity (1149.7 U/mg) against *p*NP-GlcNAc as 100%, *Sa*HEX showed moderate activity on *p*NP-GalNAc (42.7%). Among the tested CHOS of DP 2–6, *Sa*HEX showed the highest activity against $(GlcNAc)_4$, followed by $GlcNAc_5$, $GlcNAc_6$, $GlcNAc_3$, and $GlcNAc_2$. It also displayed significant activity towards colloidal chitin After 6 h of incubation with crab shell α-chitin, GlcNAc was detected in the reaction mixture (data not shown). Most HEXs of GH3, GH20, and GH84 hardly hydrolyze long chain chitin polymers. To our best knowledge, there are only two exceptions, i.e., the GH20 HEXs from *Vibrio harveyi* (31) and *Le*Hex20A from *Lentinula edodes* (shiitake mushroom) (32) that have capability of degrading chitin polysaccharide. Thus *Sa*HEX displaying broad substrate specificity might be potential for wide application.

Highly active enzymes are preferred for industrial application. However, most functionally characterized GH20 HEXs showed a specific activity of less than 1000 μmol/min/mg towards *p*NP-GlcNAc (25). The HEXs from *S. coelicolor* A3(2) (18), *Sphingobacterium* sp. HWLB1 (33) and *Vibrio alginolytius* (34) had activities of 0.37–180 μmol/min/mg towards CHOS of DP2-6. And the HEX *Vh*Nag2 from *V. harveyi* had only 1.5 U/mg against 5% of colloidal chitin (31). In comparison to these enzymes (Table S2), *Sa*HEX under study has higher activities towards synthetic (*p*NP-GlcNAc)

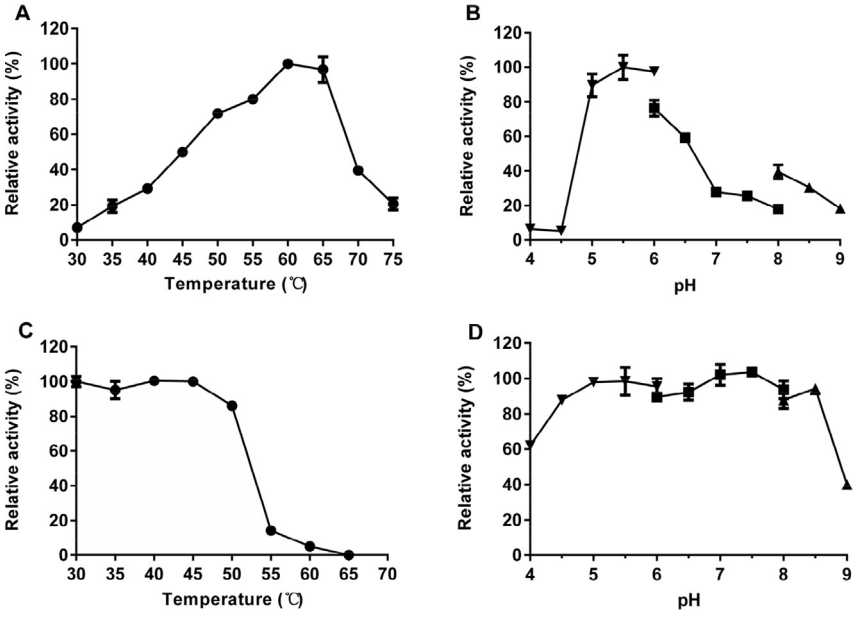

FIG. 2. Effects of temperature and pH on the *Sa*HEX activity and stability. (A) Temperature-activity profile; (B) pH-activity profile; (C) thermal stability; (D) pH stability. The buffers used were acetate buffer (downtriangles), sodium phosphate buffer (squares), and Tris/HCl buffer (triangles).

TABLE 2. Substrate specificity of the purified *Sa*HEX.

Substrate	Specific activity (U/mg)
*p*NP-GlcNAc	1149.7 ± 72.6
*p*NP-GlaNAc	491.0 ± 14.8
$(GlcNAc)_2$	215.0 ± 4.7
$(GlcNAc)_3$	234.9 ± 15.4
$(GlcNAc)_4$	395.2 ± 17.4
$(GlcNAc)_5$	253.9 ± 11.2
$(GlcNAc)_6$	242.6 ± 10.7
Colloidal chitin	4.5 ± 0.0

and natural CHOS and long chain chitin substrates, and might combine with chitinase to produce GlcNAc for industrial purposes.

The Michaelis–Menten constants K_m and V_{max} of purified *Sa*HEX were determined to be 0.79 mM and 1236.1 μmol/min/mg with *p*NP-GlcNAc as the substrate. The corresponding turnover rate (k_{cat}) and catalytic efficiency (k_{cat}/K_m) were calculated to be 1252.6/s and 1590.8 mM/s, respectively.

Action mode of *Sa*HEX TLC was performed to analyze the hydrolytic products of CHOS by the action of *Sa*HEX. The enzyme cleaved all tested CHOS substrates (DP 2–6) and released GlcNAc as the only and final product (Fig. 3). The results confirmed that *Sa*HEX was an exo-type glycosidase that catalyzes the end of the substrate to release GlcNAc.

Synergistic action on chitin Chitinolytic enzymes have been used to convert chitin; of them the enzyme combination of chitinase and HEX is essential to degrade chitin completely. For example, the combination of three *Serratia marcescens* chitinases

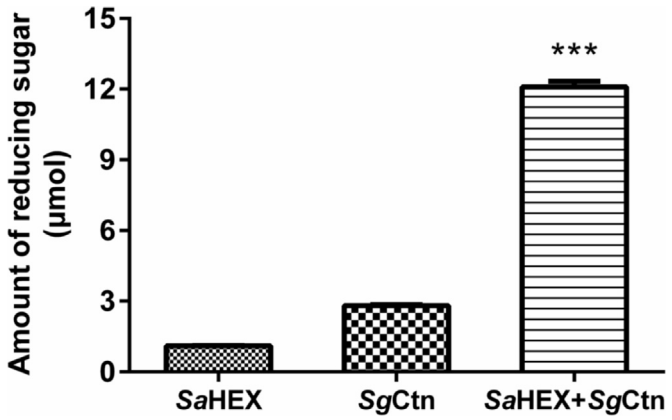

FIG. 4. Synergistic action of *Sa*HEX and a commercial chitinase *Sg*Ctn on the degradation of colloidal chitin. *Sa*HEX (50 μg/mL), *Sg*Ctn (2 mg/mL) or their combinations were incubated with 1% (w/v) of colloidal chitin in 50 mM acetate buffer (pH 5.5) at 45°C for 1 h, followed by the measurement of reducing sugar amounts released.

and the *Of*HEX from *O. furnacalis* converted 93% of the fungal mycelial waste into GlcNAc (95% purity) at 24 h (12); when using α-chitin powder as the substrate, the recombinant *Sc*HEX and chitinase C of *S. coelicolor*A3(2) achieved a conversion of 90% at 8 h (97% purity) (23); and the crude chitinases from *Aeromona scaviae* hydrolyzed 85% of colloidal chitin to GlcNAc at 6 h, and achieved 93% yield at 24 h (35).

Using colloidal chitin as the substrate, the combination of commercial chitinase *Sg*Ctn and *Sa*HEX released more reducing

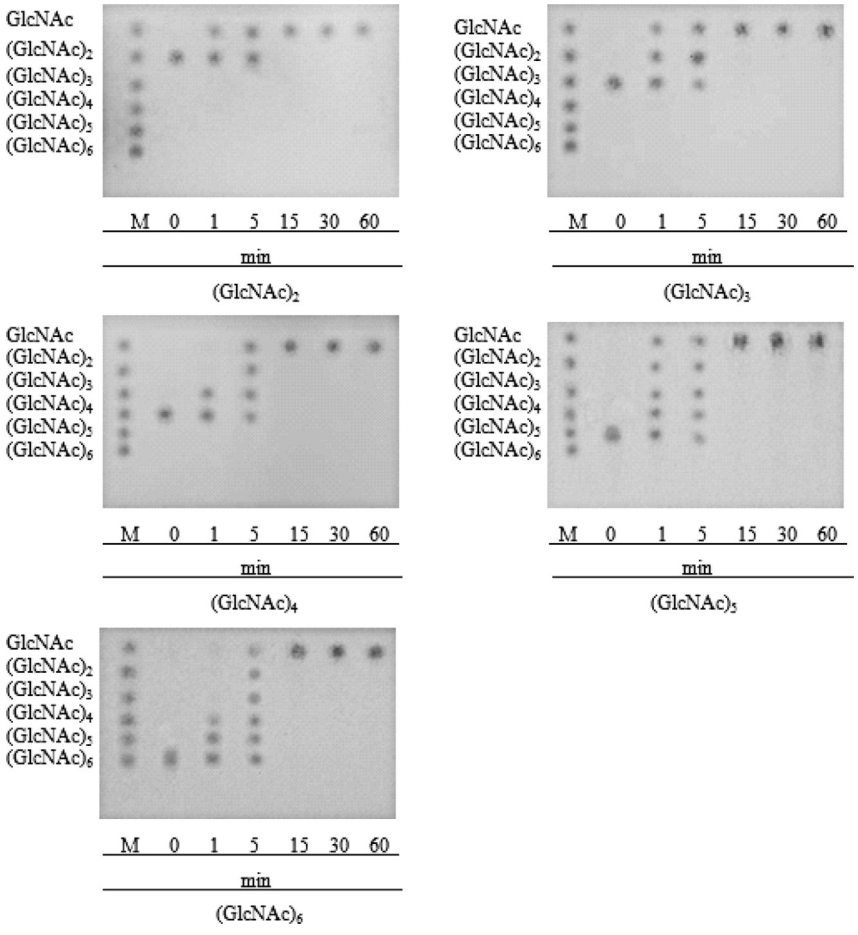

FIG. 3. TLC analysis of the hydrolytic products of $GlcNAc_n$ (n = 2–6) by the purified recombinant *Sa*HEX.

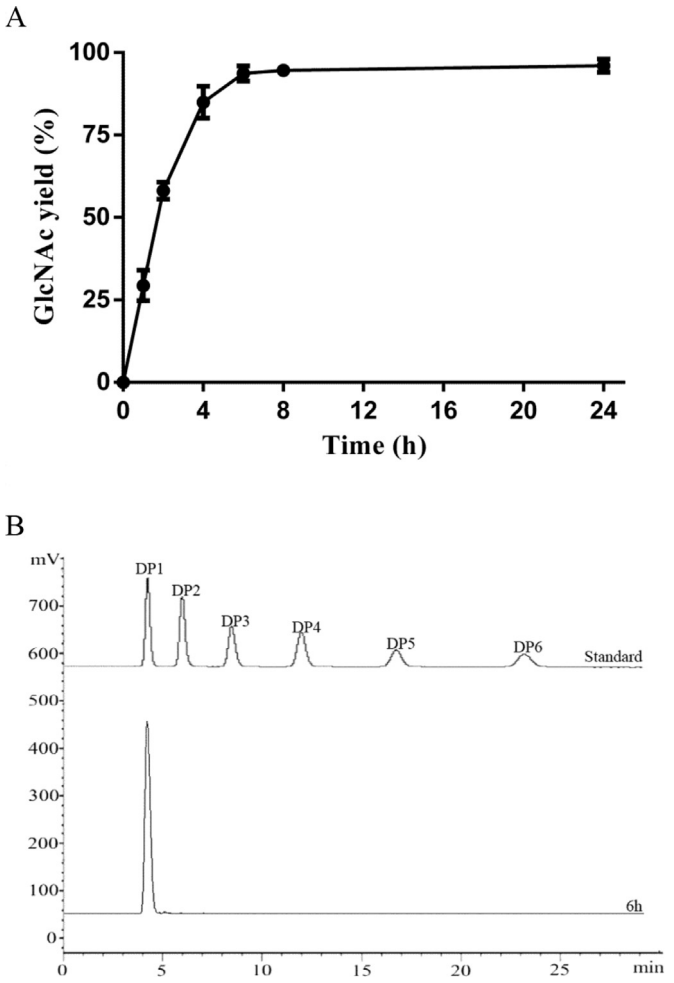

FIG. 5. Colloidal chitin hydrolysis by the enzyme combination of *Sa*HEX (50 μg/mL) and *Sg*Ctn (2 mg/mL) at 45°C in 50 mM acetate buffer (pH 5.5) for 24 h. (A) Time course of the GlcNAc production; (B) HPLC analysis of the hydrolysis products.

sugars than *Sg*Ctn (4.3-fold) and *Sa*HEX (8.1-fold) alone (Fig. 4). The synergy degree was determined to be 3.1, which is much higher than that of the binary system of *Sg*Ctn and a GH20 HEX from *Shinella* sp. JB10 (2.35) (24). It might be ascribed to the high catalytic activity and broad substrate specificity of *Sa*HEX. HPLC was then used to monitor the hydrolysis products of colloidal chitin degraded by the combination of *Sa*HEX and *Sg*Ctn over 24 h. Colloidal chitin was degraded rapidly at the beginning, and maximum conversion was achieved at 6 h, with the GlcNAc yield of 93.7%. GlcNAc was released as the unique product with the purity of >98%, and no $(GlcNAc)_2$, the final hydrolytic product of chitinase, was detected (Fig. 5). It indicated that $(GlcNAc)_2$ was completely cleaved by *Sa*HEX. Therefore, in terms of conversion rate, the chitinolytic system of *Sa*HEX and *Sg*Ctn is more efficient in GlcNAc production with the highest product purity.

In summary, a HEX, *Sa*HEX, was identified in *S. alfalfae* ACCC40021, and successfully expressed in *E. coli* BL21(DE3). The purified recombinant *Sa*HEX showed acidic, thermophilic and thermostable properties and had high catalytic efficiency, broad substrate specificity, great chitin conversion rate and product purity. These excellent properties make it potential as a biocatalyst candidate for enzymatic conversion of chitin to GlcNAc in industrial fields.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.01.017.

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