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Purification and Characterization of an Endo-*N*-Acetyl-β-D-Glucosaminidase from the Culture Medium of *Stigmatella aurantiaca* DW4

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A novel endo-*N*-acetyl-β-D-glucosaminidase (ENGase), acting on the di-*N*-acetylchitobiosyl part of N-linked glycans, was characterized in the culture medium of *Stigmatella aurantiaca* DW4. Purified to homogeneity by ammonium sulfate precipitation, gel filtration, and chromatofocusing, this ENGase presents, upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a molecular mass near 27 kDa. Optimal pH and pI were 4.0 and 6.8, respectively. The enzyme, named ENGase St, exhibits high activity on oligomannoside-type glycoasparagines and glycoproteins and could also hydrolyze hybrid- and complex-type glycoasparagines but does not acts as a murein hydrolase.

Cell surface carbohydrates, particularly those associated with glycoproteins, are known to play essential roles in cell adhesion and cell recognition phenomena (35). To elucidate the structure and function of the oligosaccharide moieties of glycoproteins, exoglycosidases and endoglycosidases are generally used. The enzymes acting on the di-*N*-acetylchitobiosyl part of N-linked glycans appear to be the most useful in determining the relation between structure and function of glycoproteins (33). These enzymes, endo-*N*-acetyl-β-D-glucosaminidase (ENGase) and peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase were qualified as the restriction enzymes of the carbohydrate world (16). Although they proved useful tools for studying glycoproteins, little attention was given to the understanding of their possible roles in the physiology of the producing cells.

We demonstrated that the myxobacterium *Stigmatella aurantiaca* DW4 produced in its culture medium an ENGase (ENGase St) acting on the di-*N*-acetylchitobiosyl part of N-linked glycans. This novel ENGase is different from the ENGases previously isolated from *Myxococcus virescens* (10) and *Myxococcus xanthus* (30) and characterized as murein hydrolases cleaving the 4-*O*-(*N*-acetyl-β-D-glucosaminyl)-*N*-acetyl-muramyl bond.

As a first attempt to investigate the possible role of the ENGase in the life of myxobacteria, we purified this enzyme and characterized its physical properties and substrate specificity.

MATERIALS AND METHODS

Materials. Polybuffer exchanger PBE 94, Polybuffer 74 for chromatofocusing, molecular weight markers, and Sephadex G-75 were purchased from Pharmacia (Uppsala, Sweden). Bio-Gel P-60 and protein assay reagent were from Bio-Rad (Richmond, Calif.). Casitone was from Difco Laboratories (Detroit, Mich.). Azocasein, RNase B, and bovine albumin were from Sigma Chemical Co. (St. Louis, Mo.). Silica Gel 60 plates and trifluoroacetic acid were from Merck (Darmstadt, Germany). Ultrafiltration membrane was purchased from Millipore Corp. (Bedford, Mass.). All other reagents were of the best quality available.

Microorganism and culture conditions. *S. aurantiaca* DW4 (23) was grown in CLM medium (6). Cultures of 20 ml in 250-ml Erlenmeyer flasks were shaken in an orbital incubator (120 cycles per min) at 30°C for 48 h. The seed culture obtained was transferred to 3-liter culture flasks, each flask containing 1 liter of medium. ENGase activity was assayed on filtered (with a 0.45-μm-pore-size membrane) supernatant.

Cell fractionation. Cells were harvested by centrifugation at 12,000 × *g* for 15 min, and the fractionation was carried out by the method of Orndorff and Dworkin (20) as modified by Hartzell and Kaiser (9). ENGase activity was assayed in the periplasmic, cytoplasmic, and membrane fractions.

Assay of the ENGase activity. The ENGase activity was detected and quantified with the resorufin-labelled $Man_7GlcNAc_2Asn$ glycoasparagine (resorufin-labelled Man_7) in the fluorescent high-performance liquid chromatography assay as reported previously (2). One unit of activity is defined as the amount of enzyme necessary to transform 1 μmol of substrate per min at 37°C in 0.1 M sodium acetate buffer (pH 4.0). If necessary, when the enzyme extract is highly pigmented, cold absolute ethanol (stored at −20°C; final concentration, 80% [vol/vol]) must be added to precipitate proteins and the mixture must be allowed to stand at 4°C and centrifuged for 5 min at 12,000 × *g*; in this way, the pellet is finally dissolved and ready to be assayed for enzyme activity.

Assay of the activity of other enzymes. Exoglycosidase activities were assayed by using the appropriate *p*-nitrophenyl glycosides (*p*-nitrophenyl α- [or β]-D-mannopyranoside and β-D-galactopyranoside) (15). The reaction mixture was incubated at 37°C for 1 h, and the A_{400} was measured. Protease activity was determined by incubating azocasein with the enzyme preparation in 0.1 M Tris-HCl buffer (pH 7.6) at 37°C for 18 h (27). The reaction was terminated by the addition of 5% trichloroacetic acid. The A_{440} of the supernatant was measured. To determine the bacteriolytic activity, the turbidity decrease in a suspension of *Micrococcus lysodeikticus* cells (Sigma) was monitored. The cells were suspended in 0.1 M sodium acetate (pH 4.0), 0.1 M sodium phosphate (pH 7.2), or

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Tris-HCl (pH 8.6) buffer. One-tenth milliliter of the test solution was introduced into 2.5 ml of the cell suspension placed in the spectrophotometer cuvette at 37°C. The A_{450} was measured immediately after mixing for 5 min. One bacteriolytic enzyme unit was defined as the amount of enzyme which gives a decrease of 0.001 absorbance units per min under the specified conditions (11).

SDS-PAGE. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (14) with 12.5% polyacrylamide gels by using a SE250 Mighty Small II (HSI, San Francisco, Calif.) electrophoresis system and stained with Coomassie blue. SDS-PAGE with 20% homogeneous PhastGel and isoelectric focusing with PhastGel IEF 3-9 were also performed with a PhastSystem (Pharmacia). The sample buffer was 0.01 M Tris-HCl (pH 8.0) containing 2.5% SDS and in some cases 5% (vol/vol) β-mercaptoethanol. PhastGels were silver stained (12). The following standard proteins were used: α-lactalbumin (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), and phosphorylase *b* (94 kDa; all from Pharmacia).

Purification of ENGase. Ultrafiltration was carried out with a Pellicon (Millipore) apparatus equipped with a PTGC 10,000 polysulfone membrane. The Bio-Gel P-60 column (2.5 by 80 cm) was equilibrated with 0.05 M Tris-HCl buffer (pH 7.5). The flow rate was 30 ml/h, and 2.5-ml fractions were collected. The chromatofocusing PBE 94 column (1 by 45 cm) was equilibrated with 0.025 M imidazole-HCl buffer (pH 7.4). The flow rate was 60 ml/h, and 4-ml fractions were collected. The column of Sephadex G-75 (1.5 by 60 cm) was equilibrated with 0.005 M sodium acetate buffer (pH 4.5). The protein content after each step was determined with a Bio-Rad protein assay kit (4), with bovine serum albumin as a standard.

Determination of N-terminal sequence. Automated Edman degradation was carried out in an Applied Biosystems model 470-A protein sequencer, and the phenylthiohydantoins of the amino acids were identified by using an on-line Applied Biosystems model 120-A phenylthiohydantoin analyzer.

Assay for optimum pH. To determine the optimum pH of ENGase St, the substrate was incubated with 20 μl of enzyme solution previously suspended in 100 mM appropriate buffer (sodium citrate for pH 2.5 to 4.5; sodium acetate for pH 3.5 to 7.0, and Tris-HCl for pH 7 to 9). The assay of the enzyme activity was then performed as described above.

Substrate specificity. Glycoasparagine and glycopeptide stock solutions (1 mg/ml of sugar content) were made in 0.1 M sodium acetate buffer (pH 4.0). Glycoasparagines of the oligomannoside type and the hybrid GPIIIA (31) glycoasparagine provided by J. C. Michalski were obtained by pronase digestion of hen ovalbumin and purified as described previously (13). Complex biantennary disialylated glycoasparagine was obtained by pronase digestion of human plasma proteins and purified by anion-exchange chromatography (17). The asialo derivative of this glycoasparagine was obtained by treatment with 0.05 M HCl for 1 h at 80°C and subsequent purification by gel filtration on a Bio-Gel P-2 column. The monosaccharide composition was determined by gas-liquid chromatography after methanolysis (methanol–0.5 M HCl; 24 h at 80°C) and trimethylsilylation (18). The complex biantennary asialo glycopeptide and the glycopeptide from bromelain obtained from F. Altmann were prepared as described previously (34). The release of oligosaccharides from the glycoasparagines due to the action of the enzyme was monitored by thin-layer chromatography performed on Silica Gel 60 plates (Art. 5553; Merck) with *n*-butanol–acetic acid–water, 2:1:1 (vol/vol/vol), for 1 h and then with ethanol–*n*-butanol–acetic acid–pyridine–water, 100:10:3:10:30 (by volume), for 4 h. The modification of the glycoproteins tested was monitored by SDS-PAGE (14).

RESULTS

Production of ENGase St during vegetative growth. The growth of *S. aurantiaca* was characterized by a log phase (20 to 50 h) and a brief stationary phase (50 to 65 h), followed by a rapid autolysis indicated by a pronounced pigmentation (24). The ENGase was assayed on filtered supernatant from growing cells. It appeared that maximum activity was reached at the end of the log phase. Because of the low production of ENGase activity in the medium, culture growth was stopped just before the autolysis, when activity was at a maximum level (0.85 mU/ml), in spite of the fact that pigments appeared when the culture growth approached the stationary phase. After fractionation of cells taken during log phase, no ENGase activity was detected either in the periplasmic fraction or in the cytoplasmic and membrane fractions. ENGase is a protein found in the culture medium and not in the cells, and it was evident that it was not a result of cell lysis. On the basis of the actual definition of secretion (8), we can consider ENGase to be a secreted enzyme.

Purification of ENGase St. Step 1. Concentration and selective precipitation. The culture filtrate (3 liters) was concentrated 10-fold by ultrafiltration. This step facilitated subsequent precipitation by ammonium sulfate but resulted in a substantial loss of the activity (24%). The retentate was brought to 80% (wt/vol) saturation by adding solid ammonium sulfate with gentle stirring. The precipitate that formed, after one night at 4°C, was collected by centrifugation and dissolved in a minimum amount of water to obtain the crude enzyme preparation.

Step 2. Gel filtration on Bio-Gel P-60. The crude enzyme preparation was applied to the Bio-Gel P-60 column. Proteins were monitored at 280 nm, and ENGase activity was assayed with the resorufin-labelled glycoasparagine (not shown). The enzyme-containing fractions were pooled. This step was useful in eliminating the pigments.

Step 3. Chromatofocusing. The enzyme fraction from step 2 was exhaustively dialyzed against 0.025 M imidazole-HCl buffer (pH 7.4) and applied to the PBE 94 column which was subsequently eluted with Polybuffer 74-HCl (pH 4.0) to generate a linear pH gradient from 7.4 to 4.0. The enzyme activity eluted in the region of pH 6.8. ENGase activity was assayed with the resorufin-labelled glycoasparagine. Fractions containing the activity were pooled and concentrated to about 1/10 of the original volume by dialysis against polyethylene glycol 20,000.

Step 4. Sephadex G-75. The concentrated enzyme was applied to a Sephadex G-75 column. Proteins were monitored at 280 nm, and ENGase activity was assayed with the resorufin-labelled glycoasparagine (not shown). After this step, the enzyme was purified about 1,000-fold from culture medium, with a yield of 57% (Table 1).

Enzyme purity and physical properties. After purification step 4, ENGase St was completely separated from contaminating proteins, and ampholytes were efficiently eliminated. By using *p*-nitrophenyl glycosides as the substrate, the enzyme preparation was found to contain no exoglycosidases. No protease activity was detectable after an overnight incubation with azocasein. No bacteriolytic activity was detected when purified ENGase St was incubated with *M. lysodeikticus* cells. The purified ENGase preparation showed a single protein band on SDS-polyacrylamide gels (Fig. 1, lane 2), and the

TABLE 1. Purification of ENGase St from the culture filtrate of *S. aurantiaca*

Purification step	Vol (ml)	Activity (mU)	Protein (mg)	Sp act (mU/mg)	Yield (%)
Culture filtrate	1,100	1,035	83.4	12.4	100
$(NH_4)_2SO_4$ precipitate	23	747	72	10.3	72
Bio-Gel P-60	18.5	715	14.4	49.7	69
Chromatofocusing and Sephadex G75	19	590	0.05	11,800	57

molecular mass was estimated to be 27 ± 0.5 kDa under reducing conditions. The pI of the protein determined by the chromatofocusing step was 6.80 ± 0.05, the value confirmed by analytical isoelectric focusing. The purity of the enzyme permitted the N-terminal sequence determination of the protein. The first amino acid was not identified (X), but the following 11 residues were identified: XNEPPLAPTGL. This sequence was compared with the N-terminal portion of other ENGases, and we noticed that three residues (Pro-4, Pro-5, and Thr-9) were identical in the ENGases from *S. aurantiaca* (ENGase St), *Streptomyces plicatus* (ENGase H) (25), and *Flavobacterium* sp. (ENGase Fsp) (32).

ENGase St appears to be stable for months when stored at 4°C, and lyophilization does not affect its activity. The effect of pH on the enzymatic activity was studied with the resorufin-labelled N-glycopeptide. Maximum activity was obtained at pH 3.0 to 5.0, which decreased to about 60% at pH 8.0. The enzyme was stable between pH 4 and 8 when kept at 4°C for 48 h.

The effect of different metal ions at 10 mM ($MgSO_4$, NH_4Cl, $MnSO_4$, $ZnCl_2$, $CuSO_4$, $CaCl_2$, $FeCl_3$, and $NaNO_3$) on the activity of the enzyme was tested. The effect of each compound was examined after a time lag of 10 min at 37°C. ENGase St was resistant to all metal ions tested in the conditions described above. Only $FeCl_3$ inactivated the enzyme significantly

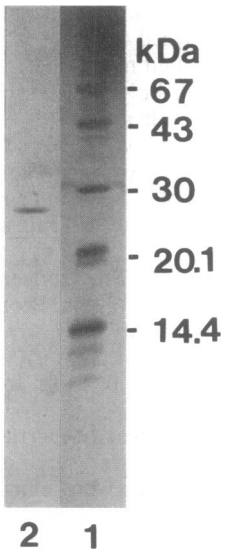

FIG. 1. SDS–12.5% polyacrylamide gel under reducing conditions of the purified enzyme after chromatography on the Sephadex G-75 column (step 4). Lanes: 1, standard proteins; 2, purified ENGase St. The gel was silver stained. Molecular mass markers are shown on the right.

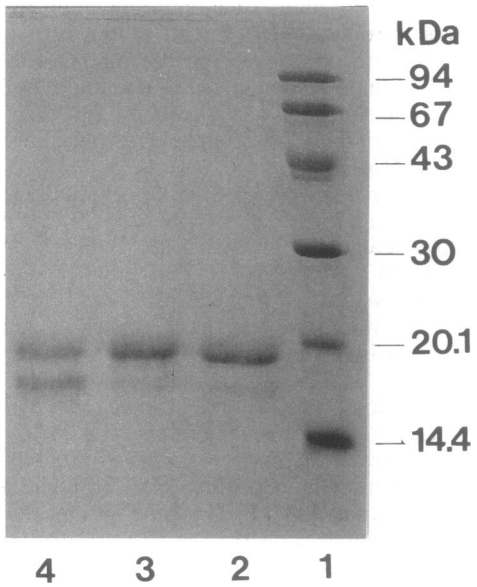

FIG. 2. Substrate specificity of ENGase St. SDS-PAGE of RNase B was done before (lane 2) and after (lanes 3 and 4) treatment with ENGase St. Lanes: 1, standard proteins; 2 and 3, native RNase B; 4, denatured RNase B. Molecular mass markers are shown on the right.

(about 50% of the original activity). Other agents such as 10% citric acid, 3 M urea, or 10% formamide inactivated the enzyme.

Substrate specificity. The release of oligosaccharides from various glycoproteins by ENGase St was tested with bovine pancreatic RNase B as the substrate. This glycoprotein contains a single high-mannose type sugar chain composed of $Man_{5-9}GlcNAc_2$, with 48% of total glycoforms having five mannose residues (26). The substrate was incubated with 4 mU of ENGase St in 0.1 M sodium acetate buffer (pH 4.0) for 2 h at 37°C. Aliquots of the enzyme and control digests were subjected to SDS-PAGE. No modification of the native glycoprotein was obtained (Fig. 2, lane 3). The molecular weight of RNase B showed a decrease of about 2 kDa after the treatment with ENGase under denaturing conditions (Fig. 2, lane 4). The transformation was completed after an overnight incubation (not shown). Ovalbumin, which contains both oligomannoside- and hybrid-type oligosaccharides (1, 37), was not cleaved under either denaturing or native conditions (not shown).

Results concerning the susceptibility of different glycoasparagines or glycopeptides to ENGase St are summarized in Table 2. ENGase St was preferentially active on glycoasparagines and glycopeptides of the oligomannoside type with five to nine mannose residues. It was also able to cleave substrates of the hybrid and complex types in the asialo form. Thus, ENGase St displays a broader specificity than ENGase H from *S. plicatus*; which is able to cleave only glycopeptide and glycoasparagine substrates of the oligomannoside and hybrid types.

DISCUSSION

ENGase was first detected in 1971 in the culture medium of *Streptococcus pneumoniae* (19). Several similar enzymes have subsequently been obtained from different microorganisms. ENGases were also found in plants, in several animal tissues, and in the culture filtrates and vegetative cells of fungi (for a review, see reference 16).

The ENGase purified from the culture medium of *S. auran-*

TABLE 2. Substrate specificity of ENGase St from *S. aurantiaca* towards glycoasparagines and glycopeptides

Glycoasparagine or glycopeptide type	Sugar content	Relative hydrolysis rate (%)[a]	Structure reference
Glycoasparagines			
oligomannoside	$Man_7GlcNAc_2Asn$	100	2
oligomannoside	$Man_9GlcNAc_2Asn$	100	36
hybrid	$GlcNAc_2Man_5GlcNAc_2Asn$	30	31
complex	$NeuAc_2Gal_2GlcNAc_2Man_3GlcNAc_2Asn$	10	17
complex	$Gal_2GlcNAc_2Man_3GlcNAc_2Asn$	10	17
Glycopeptides			
oligomannoside	$Man_5GlcNAc_2Asn$-peptide	100	3
complex	$Gal_2GlcNAc_2Man_3GlcNAc_2Asn$-peptide	10	34
complex	$(Xyl)Man_2GlcNAc(Fuc)GlcNAcAsn$-peptide	0	34

[a] The concentration of the substrates (expressed in sugar content) was 1 mg/ml. The hydrolysis rate was estimated relative to that of the Man_7 derivative.

tiaca is different from those acting on murein (10, 30) and represents the first enzyme acting on the di-*N*-acetylchitobiosyl part of N-linked glycans to be detected in a myxobacterium. This gram-negative bacterium has a complex life cycle that includes development. The developmental phenomena that mediate fruiting-body formation clearly involve major changes in global patterns of gene expression (29). Since no *N*-glycosylprotein has been unambiguously characterized in myxobacteria, corresponding to a potential substrate for the ENGase activity, the biological role of this enzyme is debatable.

To obtain small peptides and amino acids from exogenous proteins and to satisfy energy and nitrogen requirements, we can imagine that myxobacteria eliminate the oligosaccharide moieties which often protect polypeptides against peptide bond hydrolysis. For this purpose, bacteria release, in their environment, hydrolytic enzymes which decay animal and plant tissues. Until now, proteases were the main class of hydrolytic enzymes which had been identified, discrete species being characterized and sometimes purified (5, 7, 21). Recently, it has been established (22) that some secreted trypsin-like proteases from the well-known myxobacterium *M. xanthus* were a component of a factor, a complex mixture of amino acids, peptides, and proteases which induce the differentiation pathway of *M. xanthus* following starvation. The ENGase described in this study can contribute to the accessibility of the peptide part of *N*-glycosylproteins which might be enhanced by separation of the oligosaccharide and protein moieties.

Another possibility is that, in the same way as proteases do for peptides, endoglycosidases might be able to release discrete oligosaccharides from native *N*-glycosylproteins excreted by the bacterium and thus produce a family of developmental signals. Such a situation has never been described in procaryotes but was already observed during eucaryotic developmental processes. Indeed, peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase activity was found in the early embryos of *Oryzias latipes* (Medaka fish) (28), and it is thought that this enzyme releases oligosaccharides participating in the developmental process.

Thus, the biological role of ENGase St could be a means (i) to obtain amino acids and peptides more easily from foreign *N*-glycosylproteins and (ii) to release oligosaccharide moieties, which can act as biological signals.

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