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Purification and Characterization of a Novel Sialidase from a Strain of *Arthrobacter nicotianae*

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The nonpathogenic strain *Arthrobacter nicotianae* produces two sialidase isoenzymes, NA1 and NA2, with molecular masses of 65 kDa and 54 kDa, respectively, as determined by 10% SDS–polyacrylamide gel electrophoresis. NA1 and NA2 exhibit maximum activities at pH 4 and 5, and both show clear thermal optima at 40°C. They are stable at temperatures up to 50°C. The critical temperatures (T_c = 50°C and 51°C) for the two isoenzymes were determined by fluorescence spectroscopy and correlate well with the temperatures of melting (T_m = 49°C and 48°C), determined by CD spectroscopy. The isoenzymes are less stable against denaturation with Gdn.HCl, and the free energy of stabilization in water was calculated to be 7.6 and 8.0 kJ mol^{-1}, respectively. The specific activity (K_m value) toward glucomacropeptide as a substrate was calculated to be 0.126 mM for NA1 and 0.083 mM for NA2.

Key words: *Arthrobacter nicotianae*, neuraminidases, protein sequencing, sialidase EC 32.1.18, substrate specificity.

Abbreviations: CD, circular dichroism; PAGE, polyacrylamide gel electrophoresis; ultraviolet absorption; TFA, trifluoroacetic acid; Q, quantum yield.

Sialidases (neuraminidases, *N*-acylneuraminosylglycohydrolases, EC 3.2.1.18) hydrolyze sialic acids that are α-glycosidically and terminally linked to carbohydrate chains of glycoproteins, glycolipids or to oligosaccharides. These enzymes are key enzymes in the metabolism of sialic acid, as the presence of sialic acid residues in biological molecules and cell membranes decides their physiological function and fate (*1*). Highly purified sialidases are required in order to investigate more precisely the roles of sialic acids in glycoconjugates.

Sialidases are produced by a variety pathogenic microorganisms, vertebrate tissues and plasma (*2*, *3*). Two sialidase isoenzymes with molecular masses of 71 and 43 kDa were isolated from *Clostridium perfringens* spp. (*4*), and four molecular species (L, M1, M2 and S) were purified from a mutant strain of *Arthrobacter ureafaciens* (*5*). An enzyme of a membrane-associated ganglioside sialidase with a molecular mass of 52 kDa, preferentially hydrolyzing glycoproteins and oligosaccharides, was purified from bovine brain (*6*).

Physicochemical properties of this class of enzymes from various sources have been extensively studied (*5*, *7*). Comparative analysis of the primary structure of a new monomeric enzyme from *Clostridium tertium* shows 42.6% and 64.8% identical amino acids, respectively, in comparison with the sialidases from *Clostridium septicum* and *Macrobdella decora* (*3*). Comparison of repeated sequences of four sialidases, revealed that five amino acids were highly conserved at defined positions: Ser-X-Asp-X-Gly-X-Thr-Trp (*8*). Several inhibitors based on the carbohydrate compound 2-deoxy-2,3-didehydro-D-*N*-acetylneuraminic acid (DANA) have been shown to bind to this conserved active site (*9*). Crystal structures of sialidases from the influenza viruses of types A and B (*10–12*) and sialidase L from the leech *Macrobdella decora* (*13*) have been studied, and based on these studies, the enzymatic mechanism of sialidases has been suggested.

There is a special interest in using nonpathogenic microorganisms to produce this class of enzymes. Sialidase production using *Arthrobacter* (*14*), *A. ureafaciens* (*15*) and *Arthrobacter nicotianae* (*16*) is known, but the properties of the enzyme, produced by the non-pathogenic strain *A. nicotianae* have not been studied sufficiently.

The aim of this study, therefore, is to clarify the relevant properties of the two isozymes produced by the nonpathogenic strain *Arthrobacter nicotianae*. For this purpose, the isoenzymes NA1 and NA2 were isolated and characterized.

MATERIALS AND METHODS

Isolation of Sialidase—Arthrobacter nicotianae was grown as described by Abrashev *et al*. (*17*). The culture filtrate was mixed with $(NH_4)_2SO_4$ to 100% saturation, and the precipitate formed after 18 h was collected by centrifugation at 10,000 × *g* min^{-1} at 4°C for 30 min. The precipitate was dissolved in 50 mM phosphate buffer (pH 5.8) and dialyzed against the same buffer for 4 days. The enzyme was partially purified by affinity chromatogra-

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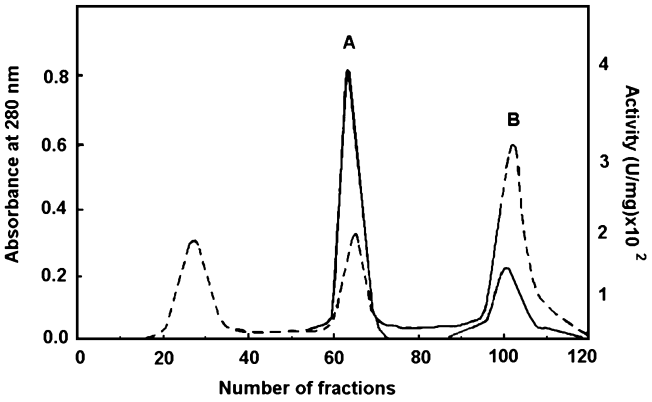

Fig. 1. **Affinity chromatography (Sepharose-glucomacropeptide 4B column, 1.2 × 20 cm) of the $(NH_4)_2SO_4$ precipitate from the crude extract.** The column was equilibrated with 0.02 M acetate buffer, pH 5.0, and eluted with a two-step gradient of 0.1 M acetate buffer, pH 5.0, containing 0.25 M NaCl, followed by 0.1 M phosphate buffer, pH 7.0. Fractions of 4 ml were assayed for activity (solid line) and absorbance at 280 nm (broken line).

phy, *via* CNBr-activated Sepharose 4B was added to a filtered solution of glycomacropeptide (containing about 5% sialic acids) in 100 mM $NaHCO_3$, 0.5 M NaCl buffer (pH 8.5). After gentle shaking for 2 h at 23°C, the Sepharose was extensively washed with 20 mM acetate buffer, containing 0.5 M NaCl (pH 5,0) and finally washed with 100 mM phosphate buffer, with 0.5 M NaCl (pH 7.0). Fractions with sialidase activity were concentrated on a Diaflo-30 instrument (Amicon, USA) with a 30 kDa membrane and were separated on a Mono S 5/5 column by a FPLC system, previously equilibrated with 250 mM acetate buffer (pH 5.0). The isoenzymes were eluted with a nonlinear NaCl gradient (0 to 0.5 M NaCl) within 60 min at a flow rate 1 ml min^{-1}, concentrated and desalted on a Sephadex G-25 column with water.

Sialidase activity was established quantitatively according to Uchida (*15*). One unit of sialidase activity (U) is defined as the amount releasing 1 g of *N*-acetylneuraminic acid in 1 min under standard conditions using glycomacropeptide as a substrate (*18*).

PAGE was carried out as described by Laemmli (*19*) on a 10% gel, and SDS- PAGE . The proteins (70 μg) were separated on gels and stained with Coomassie Brilliant Blue R-250 in 25% methanol, 10% acetic acid and water. For determination of the molecular weight, the following protein markers were used: (a) egg albumin (45 kDa), (b) bovine albumin (66 kDa); (c) phosphorylase (97.4 kDa).

Characterization of the Isoenzymes NA1 and NA2—The effect of pH and temperature on the enzyme activity was determined by determining the specific activity in buffers of various pHs and at various temperatures. Samples containing the purified enzyme NA1 with an activity of 40 U ml^{-1} and NA2 (*30* U ml^{-1}) were incubated for 30 min at 37°C in the pH range from 3.0 to 8.0 with a series of 200 mM citrate-phosphate buffers. The activity was measured and expressed as remaining sialidase activity. The temperature dependence of the sialidase activity was measured by the standard assay at pH 5.5 in the range from 25 to 60°C. Samples containing the purified enzyme with the same activity were preincubated for 20 min at each temperature, and the activity was measured and expressed as remaining sialidase activity.

Fluorescence quantum yields were determined according Kirby and Steiner (*22*) and *N*-acetyltryptophanamide (Ac-Trp-NH_2) with a quantum yield of 0.13 was chosen as a standard. The efficiency, *e*, of the tyrosine-to-tryptophan energy transfer was calculated as described by Lehrer (*20*).

The results of the quenching reactions between the excited tryptophyl side chains and acrylamide were analyzed according to the Stern-Volmer equation (*21*).

The thermostability of the enzymes was determined by different methods. After incubation of the proteins in 200 mM acetate buffer (pH 5.0) at a temperature of 25–60°C the remaining activity was determined after 2, 4, 6, 8, 24, 48, 72 and 96 h. The CD spectra were recorded with a Jasco J-720 dichrograph between 200 and 260 nm at a protein concentration of 0.2 mg ml^{-1} in 200 mM acetate buffer, pH 5.0. The data of the thermal dependence of the fluorescence quantum yield were measured with a Perkin Elmer model LS 5 spectrofluorimeter and analyzed according to Kirby and Steiner (*22*).

The denaturation in the presence of Gdn.HCl was followed using a Jasco J-720 dichrograph. The far-UV CD spectra were recorded between 200 and 260 nm at a protein concentration of 0.2 mg ml^{-1} in 200 mM acetate buffer, pH 5.0. The free energy of Gdn.HCl denaturation, $\Delta G_D^{H_2O}$, was estimated as described from Galy and Edelman (*23*).

Substrate Specificity—Activity of sialidase from *A. nicotianae* was measured using glucomacropeptide, isolated from milk whey as substrate at different concentrations. The K_m and V_{max} values were calculated from a Lineweaver-Burk plot (*24*). The novel affinity adsorbent for sialidase, Sepharose-glucomacropeptide, was shown to be easily prepared and effective for the selective purification of sialidase isoenzymes from *A. nicotianae.*

RESULTS AND DISCUSSION

Only one form with molecular mass of 69 kDa was purified from *A. nicotianae* as described by Abrashev (*17*). Using a new method, two isoforms were identified.

After precipitation of the crude extract with $(NH_4)_2SO_4$ and ultrafiltration with an Amicon PM 30 membrane, the material was adjusted to pH 5.0 and applied to a Sepharose-glucomacropeptide affinity column, pre-equilibrated with 20 mM sodium acetate buffer, pH 5.0. The elution profile is shown in Fig. 1. The first peak fraction A, which had a higher enzymatic activity (400 U ml^{-1}) and contained the NA1 and NA2 isoenzymes, was eluted as a sharp peak with 100 mM sodium acetate buffer (pH 5.0) containing 250 mM NaCl. The second protein peak fraction B, which had lower activity (100 U ml^{-1}) and contained only NA2, was eluted with 100 mM phosphate buffer, pH 7.0. Peak A was additionally separated on a Mono S 5/5 column into two isoenzymes (NA1 and NA2), leading to a 63-fold increase of specific activity. Each step of the enzyme purification was tested on 10% acrylamide gels stained for protein, and two purification steps are shown in Fig. 2. Two bands are observed in lane 2 for peak fraction A (NA1 and NA2) after separation on a Sepharose-glucomacropeptide column, and only one band

Table 1. **Purification steps for isoenzymes NA1 and NA2 from the nonpathogenic strain *Arthrobacter nicotianae*.**

Purification steps	Protein (mg)	Activity ($U.10^3$)	Spec.activity ($U\ mg^{-1}$)	Yield (fold)	Purification (%)
Crude extract	4,000.0	42,000	10.0	100.0	1.0
Precipitation with $(NH_4)_2SO_4$	125.0	22,500	180.0	53.0	17.0
Sepharose 4B column					
NA1	18.0	19,800	11,00.0	47.1	104.8
NA2	14.0	1,260	87.5	3.0	8.3
FPLC-Mono S column					
NA1	10.0	12,500	1,250.0		
NA2	6.5	650	100.0		

Table 2. **N-terminal sequences of sialidases from different sources.**

	1	5	10	15	20	25	30	35
An			L P E R Y S	**A L**	R L Q F	**D**	V D Q A I W P K S	**L** R V I Q Q
Ct	M R N L E K Y K G I I P			**A F**	Y A C Y	**D**	D E G K I S P E R	**T** Q M F T Q Y L I
Cp			M K G I Y S	**A L**	L V S F	**D**	K D G N I N E K G	**L** R E I I R H N I
Ec		M A T N L R G V M A		**A L**	L T P F	**D**	Q Q Q A L D K A S	**L** R R L V Q F N I
Hi			M R D L K G I F S	**A L**	L V S F	**N**	E D G T I N E K G	**L** R Q I I R H N I

Arthrobacter nicotianae (An); *Clostridum tertium* (Ct); *Clostridum perfringens* (Cp); *Escherichia coli* (Ec); *Haemophilus influenzae* (Hi).

(lane 3) is observed for peak fraction B (NA2). The results in Tables 1 and 2 indicate that after the last purification step, isoenzymes NA1 and NA2 were isolated as homogeneous proteins showing only one band in lanes 4 and 5 in Fig. 2, respectively. Comparison of the N-terminal sequence of *Arthrobacter nicotianae* sialidase with those of sialidases from different sources, *Clostridum tertium* (Ct), *Clostridum perfringens* (Cp), *Escherichia coli* (Ec), *Haemophilus influenzae* (Hi), shows that four positions, 13, 14, 19 and 29, are conserved (Table 2).

The molecular masses of the enzymes were determined by 10% SDS gel electrophoresis to be about 65 kDa (NA2) and 54 kDa (NA2).

Spectroscopic properties were investigated by fluorescence and CD measurements. Both methods are useful for studying folding–unfolding processes in proteins. Table 3 shows the fluorescence parameters of NA1 and NA2. Upon excitation at 295 nm, where the light is preferentially absorbed by the tryptophyl residues, the isoenzyme molecules exhibit emission spectra with maxima (λ_{max}) at 334 ± 1nm (NA1) and 338 ± 1 nm (NA2) and a tryptophyl fluorescence quantum yield of 0.036 and 0.041, respectively. These values are considerably lower than that for the model compound Ac-Trp-NH_2 (Q = 0.13), *i.e.*, for Trp in water solution. Therefore, it can be concluded that the tryptophan residues are "buried" in the molecule (*25*).

The absence of the expected tyrosyl fluorescence at 303 nm can be explained on the basis of an energy-transfer

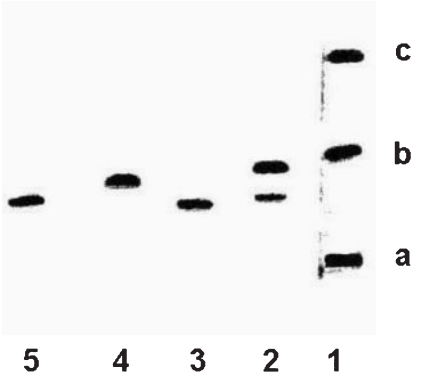

Fig. 2. **Polyacrylamide gel electrophoresis (10% gel) of purified enzymes.** Lane 1, standard mixture: (a) egg albumin (45 kDa) and (b) bovine albumin (66 kDa); (c) phosphorylase (97.4 kDa); lanes 2 and 3, peak fraction A and peak fraction B after separation on the Sepharose–glucomacropeptide 4B column; lane 4, NA1 isoenzyme; lane 5, NA2 isoenzyme.

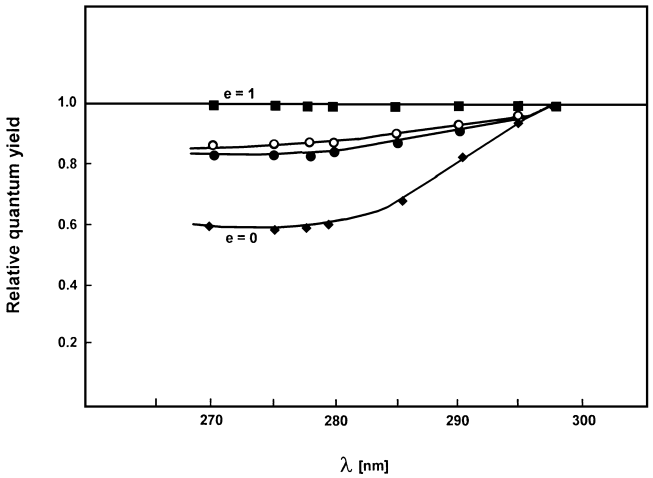

Fig. 3. **Tyrosine to tryptophan energy transfer efficiencies of the NA(open circles) and NA2 (solid circles) isoenzymes solid curves are the theoretical, obtained from different values of the transfer efficiency (e = 1.00 and 0, respectively).**

Table 3. **Properties of isoenzymes NA1 and NA2 from the non-pathogenic strain *Arthrobacter nicotianae*.**

Isoenzymes	NA1	NA2
Ea (kJ mol^{-1})	31.90	34.40
Critical temperature (T_c; °C)	50.00	50.00
Melting temperature (T_m; °C)	51.00	49.00
pH optimum	4.00	5.00
Transfer to energy (e) (%)	65.00	60.00
Quenching with acrylamide K_{SV}	11.40	9.60
KJ (K_{SV}–)	3.54	2.40
Cs^* (K_{SV}+)	1.88	1.02
Free energy $G_D{}^{H_2O}$ (kJ mol^{-1})	7.60	8.00

process involving phenol (energy donors) and indole (energy acceptors) groups (*21*). Figure 3 shows typical plots used to calculate the Tyr–Trp energy transfer efficiency, NA1 and NA2. The best fit of the experimental data to the theoretical curves was obtained for e = 0.65 and 0.60, respectively (Table 3). It can be concluded that 65% and 60% of the light is absorbed by the tyrosyl residues of NA1 and NA2, respectively, and is transferred to indole rings and emitted as tryptophan fluorescence.

The discrimination between "buried" and "exposed" residues is based on the quenching of tryptophan fluorescence by acrylamide. The quenching constants (K_{sv}) for NA1 and NA2 (Fig. 4) were calculated from the Stern-Volmer quenching plots. The quenching efficiencies K_{sv} = 11.4 and 9.6 for NA1 and NA2, respectively (Fig. 4, line 1 and 2; Table 3), are lower than K_{sv} = 16.33 M^{-1} of tryptophan in aqueous solution and indicate that the tryptophyl side chains are buried in hydrophobic regions of the enzymes. These conclusions are supported by the results of the quenching experiments with the ionic species Cs^+ and I^-. In contrast to acrylamide, which can penetrate the protein matrix, the ionic quenchers are hydrated and cannot diffuse into the protein molecule. They can quench only surface fluorophores, although complication may arise from the electric potential around the fluorophore, since the quenchers have a positive or a negative

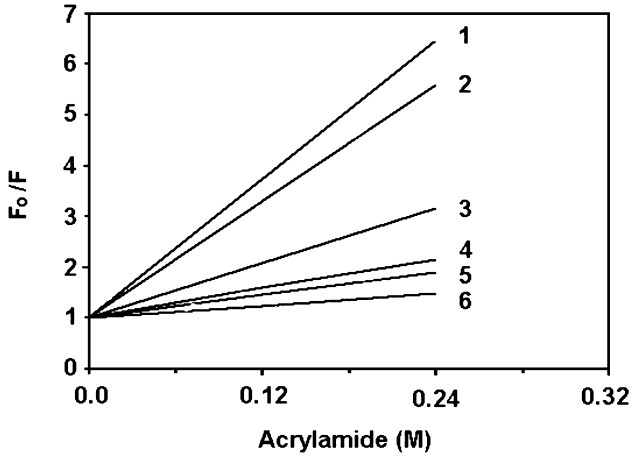

Fig. 4. **Stern-Volmer plots for fluorescence quenching were measured at 25°C after excitation of the samples at 295 nm with acrylamide: isoenzymes NA1 (line 1) and NA2 (line 2); with KJ: isoenzymes NA1 (line 3) and NA2 (line 4); and with Cs^+: NA1 (line 5) and NA2 (line 6).**

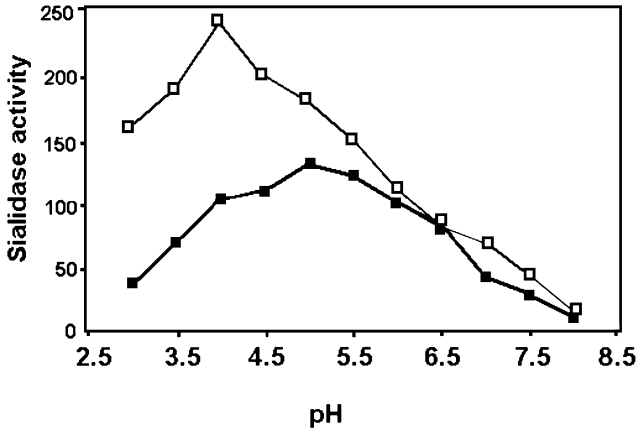

Fig. 5. **pH dependence of the activity of the NA1 (open squares) and NA2 (solid squares) isozymes.** The purified enzymes NA1 (40 U ml^{-1}) and NA2 (30 U ml^{-1}) were incubated with a series of 200 mM citrate-phosphate buffers in the pH range from 3.0 to 8.0 for 30 min at 37°C. The activity was measured and expressed as % remaining sialidase activity.

charge. The quenching constants of the ionic species for isoenzymes (Table 3; K_{sv} = 1.88 M^{-1} (NA1) and 1.02 M^{-1} (NA2) for Cs^+ and 3.54 M^{-1} (NA1) and 2.40 M^{-1} (NA2) for I^-, respectively) are lower than those for free tryptophan (2.0 M^{-1} for Cs^{2+} and 8.8 M^{-1} for I^-). These data are again consistent with the conclusion that the tryptophyl side chains are "buried" in the enzyme.

The far-ultraviolet (UV) CD spectrum of the sialidase, reflecting the backbone conformation of the protein molecule, shows two negative bands at 208 and 222 nm, which are characteristic of an α-helix structure. β-Sheets also contribute to the ellipticity around 222 nm, though to a lesser extent than α-helices. The mean residue ellipticities at 222 nm are 7,200 and 7,600 deg cm^2 dmol^{-1} for NA1 and NA2, respectively, and are typical of an α-helical structure.

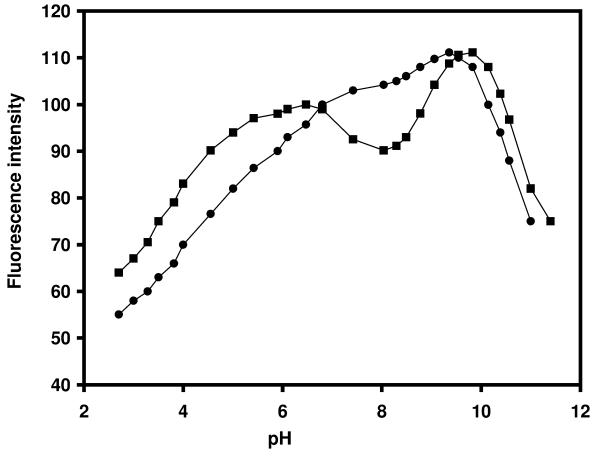

Fig. 6. **pH stability of the NA1 (solid squares) and NA2 (solid circles) isoenzymes.** The purified isoenzymes NA1 (40 U ml^{-1}) and NA2 (30 U ml^{-1}) were incubated with a series of 200 mM citrate-phosphate buffer in the pH range from 3.0 to 8.0 for 2, 4, 6, 8, 24, 48, 96 h at 37°C. The activity was measured and expressed as % remaining sialidase activity.

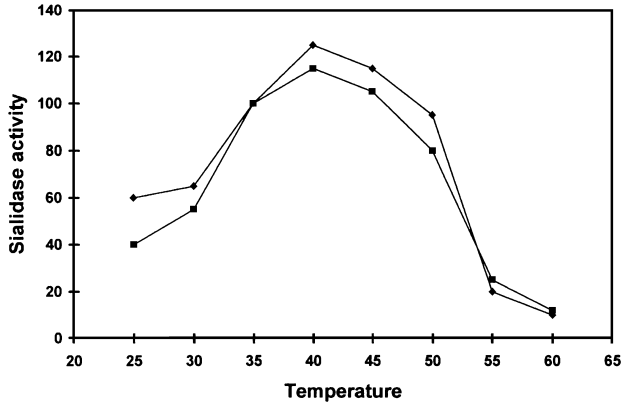

Fig. 7. **Stability of isozymes from *A. nicotianae* as a function of temperature.** The purified isoenzymes NA1 (solid circles) (40 U ml^{-1}) and NA2 (solid squares) (30 U ml^{-1}), dissolved in 0.2 M acetate buffer, pH 5.5, were incubated at the indicated temperatures for 20 min. Aliquots were withdrawn, diluted, and assayed for the remaining sialic activity.

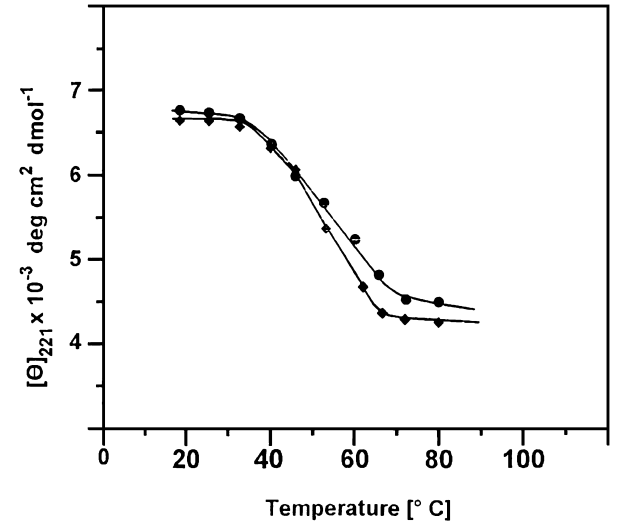

Fig. 8. **Conformational stability toward thermal denaturation of isoenzymes NA1 (solid circles) (40 U ml^{-1}) and NA2 (solid diamonds) (30 U ml^{-1}) in 0.2 M acetate buffer, pH 5.5, as estimated by CD spectroscopy.**

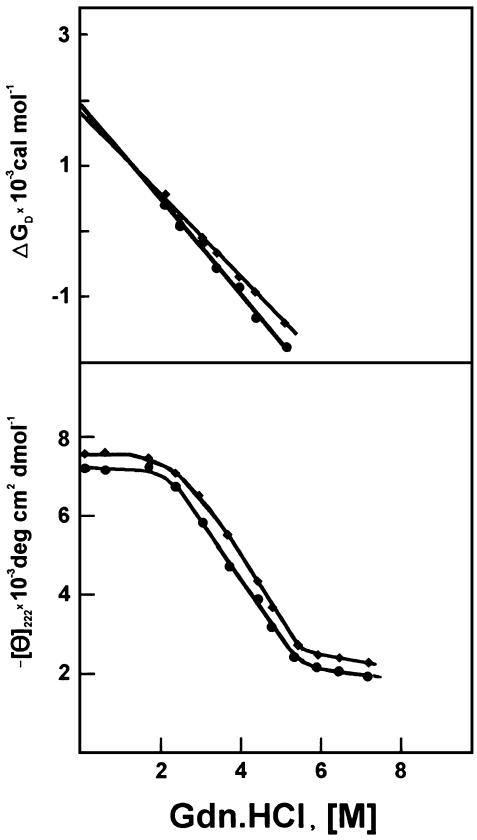

Fig. 9. **Change of the free energy ΔG_D (A) and mean residue ellipticity [Θ] at 222 nm (B) of isoenzymes NA1 (solid diamonds) and NA2 (solid circles) as a function of Gdn·HCl concentration.**

Both the activity and stability of NA1 and NA2 were tested in the pH range between 3.0 and 8.0, where pH optima around pH 4.0 and 5.0, respectively, were found for NA1 and NA2 (Fig. 5). More than 83% of the maximal activity remained between pH 3.0 and 6.0. Similar pH stability profiles were observed for enzymatic activities after 24 h of incubation at 37°C, using glucomacropeptide as a substrate. A glucomacropeptide was isolated from the milk whey, and its molecule was found to contain galactosamine, galactose and neuramic acid in a ratio of 1:2:1. The amino acid composition was determined and a molecular mass of 6,080 Da was calculated (*26*). The profiles in Fig. 6 are principally similar to the activity curves in Fig. 5. These findings are in good agreement with those of sialidases isolated from *Micromonospora viridifaciens* with a pH maximum at 5.0 (*7*), *Clostridium tertium* (pH maximum 5.5) (*3*), and *Clostridium perfringens* isoenzymes (pH maximum 6.1) (*27*). The reported optimum for sialidases from different sources is in the pH range of 4.5 to 6.5 (*5*, *28*).

The temperature dependence of the activity of NA1 and NA2 for the substrate glucomacropeptide was tested in the range of 25–60°C. As Fig. 7 shows, the activity of both isoenzimes increased up to 40°C. Different results were observed for the enzyme from *M. viridifaciens* (*7*), where the activity increased up to 58°C; and for the enzymes from *A. ureafaciens* (*5*, *3*) with temperature optima at 50°C; and for the enzyme from *C. perfringens* (51°C for the DSM and 55°C for A99 enzyme) (*27*). For most sialidases, the optimum temperature is around 37°C, which correlates with our isoenzymes.

The long-term thermostability of the isoenzymes is shown in Fig. 7. A loss of activity of about 30% was detected at a temperature of 50°C after heating of the protein samples for 30 min. A similar behaviour was observed for a sialidase from *C. perfringens* with a maximal activity around 37°C (*27*). The sialidases isolated from *A. ureafaciens* and *M. viridifaciens* (*7*) are more stable (up to 60 and 50°C, respectively) than our enzyme.

The thermostabilities of isoenzymes NA1 and NA2 were also investigated by fluorescence spectroscopy. We used the T_c-value, the critical temperature for deviation of the Arrhenius plot, $\ln (Q^{-1} - 1)$ *vs.* $1/T$, from linearity, to

Table 4. **Kinetic parameters for isoenzymes NA1 and NA2 from *Arthrobacter nicotianae* using glucomacropeptide as substrate, and comparison of sialidases from different sources: *Arthrobacter ureafaciens* (Au); *Arthrobacter ureafaciens* M1057 [Au M1057, (5)]; *Corynebacterium ulcerans* [Cu, (29)]; *Clostridium perfringens* [Cp, (26)]; *Micromonospora viridifaciens* [Mv; (7)].**

Enzymes	(Isoenzymes)	Substrate	K_m (mM)
Au	NA1	glucomacropeptide	0.126
	NA2	glucomacropeptide	0.083
Au M1057	L	[α-(2-6)-NAN-lac]	0.800
	M1	[α-(2-6)-NAN-lac]	0.600
	M2	[α-(2-6)-NAN-lac]	0.700
	S	[α-(2-6)-NAN-lac]	0.600
Cu		sialyllactose	0.520
Cp	DSM 756 NL	Mu-Neu5Ac	0.078
	A99 NL	Mu-Neu5Ac	0.075
	A99 NS	Mu-Neu5Ac	0.190
	A99 CS	Mu-Neu5Ac	0.180
Mv		colonic acid	2.100
		NANA-lactose	0.300

characterize the thermostability. The deviation indicates that at the temperature above T_c, the protein undergoes denaturation. According to the plots the isoenzymes are conformationally stable up to about 50°C.

The conformational stability against thermal denaturation of the enzymes was studied using CD spectroscopy. Figure 8 shows the temperature profiles indicating melting temperatures (T_m) of 51°C and 49°C, respectively. These results correlate well with the results obtained from fluorescence spectroscopy.

The stability of isoenzymes NA1 and NA2 against different concentrations (0–8 M) of Gdn.HCl was followed by CD spectroscopy. Figure 9 shows the influence of increasing concentration of Gdn.HCl on the intensity of the negative Cotton effect at 221 nm. A sharp decrease of the ellipticity was observed from 2.2 M Gdn.HCl with a midpoint of about 3.8 M Gdn·HCl (Fig. 9). Full denaturation and typical spectra for unfolded proteins were observed from 5.0 M Gdn·HCl. The denaturated proteins do not revert to the original conformation upon removal of the denaturation reagent.

The quantitative measure of the stability in water is the free energy of stabilization ($\Delta G_D^{H_2O}$). The free energy change associated with the transition between the globular and the random coil conformation in the absence of denaturant for NA1 and NA2 is included in Table 3. The values are obtained by linear extrapolation of ΔG_D to zero molar denaturant. These data confirm that the isoenzymes are not very stable against the Gdn·HCl denaturation. These results correlate well with the temperature denaturation experiments.

The two isoenzymes behaved quite differently on incubation with the substrate glucomacropeptide, purified from milk whey (*18*). NA1 and NA2 showed normal Michaelis-Menten behaviour with this substrate, giving a linear dependence in the Lineweaver-Burk plot (*24*). As Table 4 shows, the specific activities of NA1 and NA2 are close to the specific activity of the sialidases from *Clostridium perfringens* isoenzymes (Cp) (*27*) toward Mu-Neu5Ac as a substrate and about 5–8 times higher than those of the other enzymes included in Table 4. The two isoenzymes isolated from the *A. nicotianae* strain show different specific activity toward the substrate glucomacropeptide. Isoenzyme NA1 is 1.5 times less active against the glucomacropeptide substrate (K_m = 0.126 mM) than NA2 (K_m = 0.083 mM), confirming that our substrates is more efficient than other substrates used and that NA1 and NA2 hydrolyze sialic acids of colominic acid faster than other enzymes.

Sialidases are glycosidases which selectively digest sialic acid in carbohydrate chains connected to *O*-linked sites (-Ser/Thr-Xxx-Ser/Thr-) of protein or sialic acids in *N*-linked oligosaccharide chains. Three glycopeptides isolated from the arthropodan hemocyanin of *Carcinus aestuarii* were treated with the isoenzyme NA1 and their masses were measured by MALDI-MS. After treatment of glycopeptide 1 with sialidase to remove sialic acid, no reduction in molecular mass was observed by MALDI-MS. The same procedure was applied to glycopeptides 2 and 3, treatment of which with sialidase decreased their molecular masses, indicating the presence of 734- and 633-Da sialic acid residues corresponding to (*N*-acetyl-*O*-di-NeuAc)$_2$ and (*N*-acetyl-*O*-NeuAc)$_2$, respectively (*29*).

In conclusion, NA1 and NA2 are two new isoenzymes isolated from *A. nicotianae* with spectroscopic properties close to those of other sialidases. Most microorganisms which form sialidases are human pathogens, and the enzymes are implicated in the disease process. Our microorganism is not pathogenic and its sialidases show higher catalytic activities than other sialidases and lower K_m values toward glucomacropeptide as a substrate. These properties mean that the two isoenzymes may be suitable for optimal application in the field of glycobiology and glycotechnology.

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