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Characterization of a Novel *N*-Acetylneuraminate Lyase from *Staphylococcus carnosus* TM300 and Its Application to *N*-Acetylneuraminic Acid Production

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ABSTRACT: The possibility of incorporating *N*-acetylneuraminic acid (Neu5Ac) in infant formulas and other functional foods has opened up the need to synthesize *N*-acetylneuraminic acid using *N*-acetylneuraminate lyases (NALs) by reversible aldol condensation of pyruvate and *N*-acetyl-D-mannosamine. Until now, NALs have been cloned from pathogenic microorganisms; however, this Article describes the expression and characterization of an *N*-acetylneuraminate lyase from the *Staphylococcus carnosus* TM300, a GRAS microorganism used in fermented meat. ScNAL showed a high level of expression in *E. coli* (403 mg L^{-1} culture). This, combined with its simple two-step purification procedure, the highest recovery described to date (86%), its kinetic parameters, which are in the same order of magnitude as best reported NALs, and its optimum pH and temperature, make ScNAL a promising and cheap biocatalyst. To confirm its biotechnological potential, the Neu5Ac was synthesized in 3 h in simple industrial working conditions with a high degree of conversion (94%).

KEYWORDS: *N-acetylneuraminate lyase, N-acetylneuraminic acid, Neu5Ac synthesis, kinetic parameters, GRAS microorganism*

INTRODUCTION

N-Acetylneuraminic acid (Neu5Ac) is a key monosaccharide unit in brain gangliosides and glycoproteins, including poly-*N*-acetylneuraminic acid glycotope on neural cell adhesion molecules (NCAM), whose function in the CNS is to regulate cell migration, neurite outgrowth, axon elongation, and synaptic formation and plasticity.[1,2] Neu5Ac concentrations in brain cortical tissues are correlated with evolutionary development in higher animals, and the human brain contains twice as much Neu5Ac as the chimpanzee brain.[3] Importantly, the concentration of Neu5Ac in the frontal cortex of breast-fed infants is higher than in formula-fed infants.[4] In addition, the levels of glanglioside *N*-acetylneuraminic acid correlated significantly with ganglioside ceramide docohexeanoic acid and total ω-3 fatty acids in breast-fed infants, but not in formula-fed infants. This structural and functional link between docohexaenoic acid (DHA) and Neu5Ac may benefit early development and cognition.[4] Taken together, these findings suggest that the increase in Neu5Ac in breast-fed infants is due to the exogenous dietary supplementation of Neu5Ac, which is found in human milk but not in infant formulas based on cow milk or protein hydrolysates.[5] Thus, premature infants, who have increased in number as a result of advances in reproductive technologies and who receive artificial feeding, are likely to be at greater risk of neural deficit. However, one of the main barriers to using Neu5Ac in enriched foods is its high price, which has led the pharmaceutical industry to restrict its use in preparing advanced intermediates for antivirals, such as Relenza (GSK).[6]

The enzyme *N*-acetylneuraminic acid aldolase or *N*-acetylneuraminate lyase (NAL, EC 4.1.3.3) is a class I aldolase that catalyzes the cleavage of *N*-acetylneuraminic acid to pyruvate and *N*-acetyl-D-mannosamine, with an equilibrium that favors Neu5Ac cleavage. NAL also catalyzes the reverse aldol condensation reaction and has been used in this way to synthesize *N*-acetylneuraminic acid and some of its derivatives from pyruvate and *N*-acetyl-D-mannosamine.[7,8] However, most NALs described to date come from human pathogens, except for the NAL cloned from the commensal *Lactobacillus plantarum*.[9] This enzyme is crucial to the pathogenic species for using the carbon sources present in the mucus-rich surfaces of the human body.

In the search for new NALs from GRAS microorganisms, *Staphylococcus carnosus* was selected, because it plays a central role in the generation of the overall flavor of fermented sausages. This Article describes its cloning and characterization from the *nanA* gene of *Staphylococcus carnosus* TM300. Its high expression, easy purification procedure (with the highest recovery/yield reported in the literature), optimal kinetic characteristics, and the high degree of conversion (94%) reached in synthesizing Neu5Ac from *N*-acetyl-D-mannosamine and pyruvate make it a promising biocatalyst to reduce the production costs of Neu5Ac for use in the food industry.

MATERIALS AND METHODS

Strains, Plasmids, and Chemicals. *Staphylococcus carnosus* TM300 was from the DSMZ collection (DSM 4600). Plasmid pET28a was from Novagen (EMD Bioscience Inc., Madison, WI). The DNeasy tissue extraction kit, QIAquick PCR purification kit, and QIAprep spin miniprep kit were from Qiagen (Valencia, CA). *Pfu*

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DNA polymerase was from Stratagene (La Jolla, CA). Restriction enzymes were obtained from New England Biolabs (Berverly, MA). Sugars were from Carbosynth (Berkshire, UK). Other reagents were from Sigma-Aldrich (Madrid, Spain).

Cloning of the ScNAL Gene. The cloning and transformation techniques used were essentially those described elsewhere.[10] *Staphylococcus carnosus* TM300 cells were collected from an overnight culture of trypticase soy yeast extract medium and provided the genomic DNA to isolate *N*-acetylneuraminic acid lyase gene, denoted *nana* (Uniprot code: B9DIJ2). The 882 bp gene was amplified by PCR using the forward primer 5′-TATGGCTAGCATGGAAGAAAATTT-GAAAGGAT-3′ (*Nhe*I restriction site is underlined) and the reverse primer 5′-GCGCTCGAGTTACAAATTGTATTTATTTACTAAC-3′ (*Xho*I restriction site is underlined). The resulting PCR product was purified and digested with *Nhe*I-HF and *Xho*I restriction enzymes. The digested PCR product was ligated with predigested pET-28a vector and transformed into electrocompetent *E. coli* DH5α cells. A selected clone harboring the correct sequence, denoted pET28-*ScNAL*, was transformed into *E. coli* Rosetta (DE3) *pLys* competent cells (Novagen, WI).

Enzyme Expression and Purification. The above *E. coli* cells harboring the recombinant plasmid pET28-*ScNAL* were grown for 4 h at 37 °C in 400 mL of lysogeny broth (LB) containing kanamycin (50 μg mL^{-1}) and chloramphenicol (34 μg mL^{-1}) (LB kan-chlor) before being transferred to a 5 L fermenter (Sartorius Stedim, Barcelona, Spain), containing 4 L of Terrific Broth (Condalab, Madrid, Spain) supplemented with the above-mentioned antibiotics. This culture was allowed to grow for 3 h at 37 °C, and then induced by adding 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 12 h at 30 °C with constant stirring and oxygenation. The culture was diafiltered through a 500 kDa membrane (GE Healthcare, Uppsala, Sweden) and cleaned with 50 mM phosphate buffer pH 8.0. Cells were disrupted in 50 mM phosphate buffer pH 8.0 (550 mL) using a homogenizer (MiniZetaII, Netzsch, Selb, Germany), and the cell debris was harvested by centrifugation (6000*g*, 20 min). The recovered supernatant (crude extract, 540 mL) was treated with 3 U/mL DNase I (Sigma-Aldrich) to remove nucleic acids, and then centrifuged for 20 min at 6000*g*.

The purification was performed in two steps, starting with tangential ultrafiltration with a 50 kDa cutoff membrane on a QuixStand system (GE Healthcare). The resulting retentate (244 mL) was purified by Ni^{2+}-chelating affinity chromatography (ÄKTA Prime Plus, GE Healthcare) in a HiPrep column (GE Healthcare.). The bound enzyme was eluted with a linear imidazol gradient up to 250 mM in 50 mM phosphate buffer pH 8.0. The fractions containing the aldolase activity were pooled, desalted, concentrated, and stored at −20 °C with 10% glycerol.

The protein concentration was determined using Bradford's reagent (Bio-Rad) and bovine serum albumin (BSA) as standard. Gel filtration (Superdex 200 10/300 GL, GE Lifesciences) was used to confirm the homogeneity and the molecular weight of the purified enzyme. The column was equilibrated in 50 mM phosphate buffer pH 7.0, containing 0.15 M NaCl.[9] The molecular mass as seen by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was determined using 12% acrylamide gel. Molecular weight was also measured by liquid chromatography−mass spectrometry with electrospray ionization, using an HPLC/MS/ESI system (Agilent Technologies, Santa Clara, CA), following previously published methods.[11]

Enzyme Assay. The aldolase hydrolytic reaction was monitored both spectrophotometrically and chromatographically (HPLC). The first method is based on a coupled enzyme assay in which the pyruvate produced by the hydrolysis of Neu5Ac by NAL is reduced to lactate by lactate dehydrogenase (LDH), with the concomitant oxidation of NADH to NAD^+, which is followed spectrophotometrically at 340 nm (ε = 6300 M^{-1} cm^{-1}). The standard reaction medium (1 mL) for the above assay, which was carried out in a Shimadzu UV-2401-PC spectrophotometer, contained 150 μM NADH, 0.5 U LDH, 10 mM Neu5Ac, and 4 μg of purified ScNAL in 20 mM phosphate buffer pH 7.0. A control assay without Neu5Ac was also carried out to determine the presence of any other NADH-consuming enzymes. This hydrolytic activity was also measured from the increase in the ManNAc peak area, using an HPLC-ELSD-II (Shimadzu, Duisburg, Germany), an Amino-UK column (Imtakt Co., Kyoto, Japan), and a mobile phase (58% acetonitrile:42% 50 mM ammonium acetate) running at 0.4 mL/min at 60 °C.[9] In these conditions, the retention times (R_T) for Neu5Ac and ManNAc were 10.3 and 4.2 min, respectively. One unit of activity was defined as the amount of enzyme required to cleave 1 μmol of Neu5Ac, release 1 μmol of ManNAc in 1 min (HPLC), or consume 1 μmol of NADH in 1 min at pH 7.0 and 37 °C.[9] The synthetic reaction was followed using the above HPLC conditions, and the standard reaction medium contained 300 mM ManNAc, 50 mM pyruvate, and 20 μg of purified ScNAL in 20 mM phosphate buffer pH 7.0. One enzymatic unit was defined as the amount of enzyme required to synthesize 1 μmol of Neu5Ac per minute under the above conditions.

Stability Assays. The pH−stability was assayed by incubating the enzyme at various pH's (5.0−9.0) at 37 °C and measuring its activity by HPLC in the standard reaction medium. The heat−stability was assessed by incubating the enzyme at different temperatures (25−80 °C) in a PCR thermocycler (TGradient, Biometra, Goettingen, Germany). Aliquots (100 μL) were taken at different times, cooled on ice, and measured spectrophotometrically in the standard reaction medium.

Gel filtration-purified ScNAL was used to determine protein unfolding (melting curves) in the presence of the fluorescent dye Sypro Orange (Sigma-Aldrich). Sypro Orange is a hydrophobic, environmentally sensitive fluorophore that sharply increases its fluorescence emission when bound to hydrophobic residues exposed to the solvent, as typically occurs during protein unfolding. Thermally induced unfolding is an irreversible process that follows a typical two-state model with a sharp transition between the folded and unfolded states, where T_m is defined as the midpoint of the protein unfolding transition temperature. The T_m values obtained with this method correlate well with those obtained by other biophysical methods such as circular dichroism (CD) or differential scanning colorimetry (DSC).[12] The assay was carried out in Milli-Q water or buffer containing 10X Sypro Orange (emission at 530 nm and excitation at 490 nm), using a 7500 Fast RT-PCR machine (Applied Biosystems, Carlsbad, CA). The time/temperature control of the PCR apparatus was adapted by performing 70 steps of 1 min each and raising the temperature by 1 °C each step, from 20 to 90 °C.[9] This technique was also used to determine pH−stability. T_m values were obtained after three repeated experiments.

Synthesis of Neu5Ac. It was synthesized from *N*-acetyl-D-mannosamine and pyruvate in a reaction medium containing 0.55 M ManNAc, 1.05 M pyruvate, and different ScNAL concentrations (0.25−1 mg/mL). This reaction was also studied by repetitive feeding with lower pyruvate concentrations.[13] Thus, when the reaction reached equilibrium, 0.35 M pyruvate was added three times.[6] The reaction temperature was kept at 25 °C, and the pH of the reaction was adjusted to 7.2 with NaOH. Buffer was not used to simulate the industrial process. Samples (50 μL) were analyzed by HPLC as described above.

In Silico Analysis. Basic Local Alignment Search Tool (BLAST) searches were used to identify homologues of *N*-acetylneuraminate lyase[14] by using functionally characterized NALs from *Haemophilus influenzae*, *Clostridium perfringens*, *Lactobacillus plantarum*, and *E. coli* K-12 (Uniprot codes: P44539, Q9S4K9, P59407, and P0A6L4, respectively). The sequences were aligned using ClustalW[15] and ESPript.[16] Protein sequences were 3D modeled with Geno 3D.[17]

RESULTS AND DISCUSSION

Amino Acid Sequence Analysis. The functionally characterized NALs from *Haemophilus influenzae*, *Clostridium perfringens*, *Lactobacillus plantarum*, and *E. coli* K-12 were used as templates to find new NALs from GRAS microorganisms using the BLAST program. Among the sequences found, the deduced amino acid sequence of the *S. carnosus* TM300 *N*-acetylneuraminate lyase (ScNAL; Uniprot code B9DIJ2) showed significant identity. The sequence alignment indicated

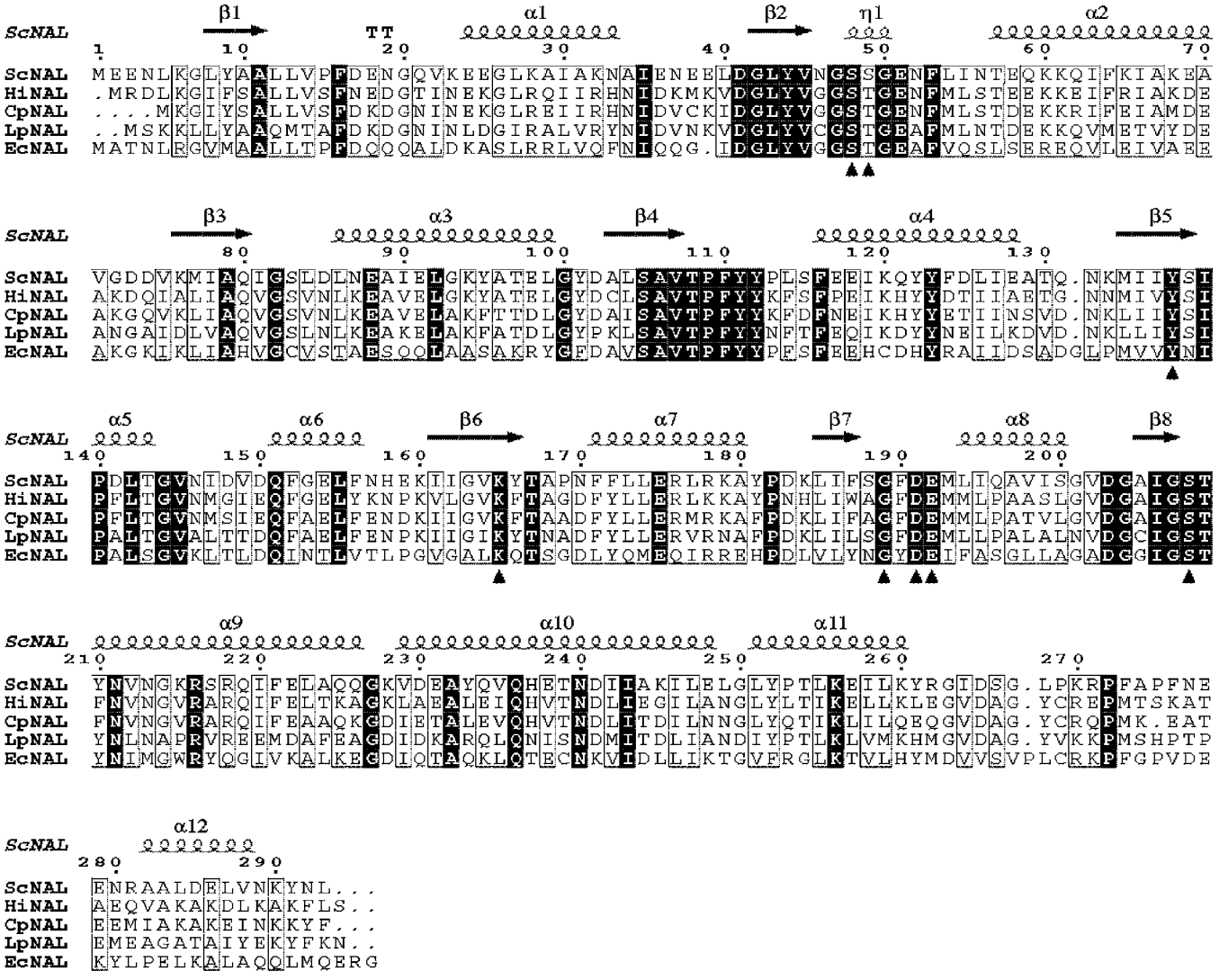

Figure 1. Multiple sequence alignment of *Staphylococcus carnosus* TM300 NAL (ScNAL) and related *N*-acetylneuraminate lyases. The ESPript output[16] was obtained with the sequences from the NCBI and aligned with CLUSTAL-W.[15] Sequences were grouped according to similarity. The enzyme showed 58% sequence identity with NAL from *Haemophilus influenzae* (HiNAL), 56% with NAL from *Clostridium perfringens* (CpNAL), 48% with *Lactobacillus plantarum* (LpNAL), and 35% with *E. coli* K-12 (EcNAL). Residues strictly conserved across NAL enzymes have a dark background. Symbols above blocks of sequences represent the secondary structure, springs represent helices, and arrows represent β-strands. The residues forming the active site are indicated by "▲".

that ScNAL has 58%, 56%, 48%, and 35% identity with NALs from *Haemophilus influenzae*, *Clostridium perfringens*, *Lactobacillus plantarum*, and *E. coli* K-12 (Uniprot code: P44539, Q9S4K9, P59407, and P0A6L4), respectively. ScNAL is more closely related to *Haemophilus influenzae* NAL (PDB code: 1F5Z) than to the other crystallized NAL from *E. coli* (PDB code: 1NAL), as calculated by Geno3D.[17] In addition, sequence alignment (Figure 1) revealed that ScNAL contained the conserved residues forming the characteristic active site of the NAL subfamily (Figure 1, ▲): the catalytic lysine at position 165 (K165, ScNAL numbering), a tyrosine at position 137 (Y137), and the conserved specific substrate (Neu5Ac) binding motif, which includes both the GxxGE motif and an another group of three amino acids (D191, E192, and S208), involved in carbohydrate moiety binding. The GxxGE motif, situated between positions 47 and 51 (ScNAL numbering), is involved in binding the carboxylate group of the α-keto moiety of the substrate, and the xx is usually S and/or T. These last two amino acids, together with Y137 and a water molecule, are involved in the hydrogen-bond network with pyruvate.[18]

Overexpression and Purification of ScNAL. *Staphylococcus carnosus* TM300 *nanA* gene was cloned into pET28a vector, which provides His_6-tagged recombinant protein (see experimental procedures). The DNA sequence of the inserted gene showed no mutations as compared to the *nanA* gene sequence reported for *S. carnosus* TM300 (GenBank accession: YP_002635477.1). The recombinant clone with the highest expression rate was induced with 1 mM IPTG in a 5 L-fermenter at 30 °C for 12 h with vigorous stirring and oxygenation. The ScNAL was purified from *E. coli* cells by a two-step procedure consisting of a 50 kDa ultrafiltration step followed by Ni^{2+}-chelating affinity chromatography in a HisTrap FF column. After these two steps, the enzyme was pure, as shown in SDS-PAGE (Figure 2, lane 3), obtaining a 7-fold purification (Table 1).

This two-step purification procedure represents a significant improvement over other methods described for NAL

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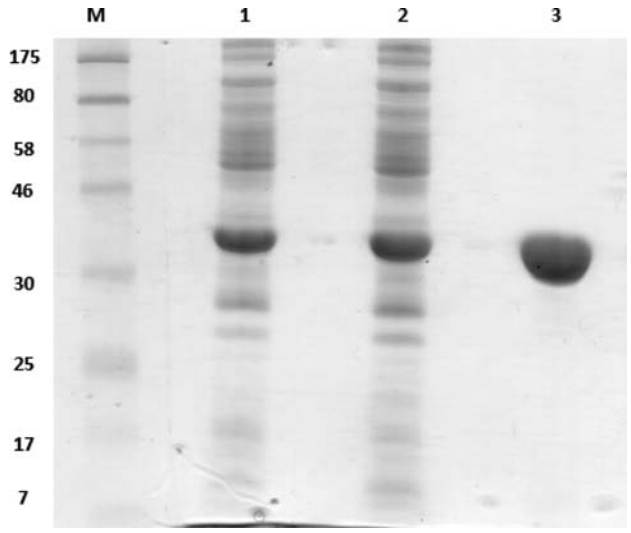

Figure 2. SDS-PAGE of the ScNAL gene product obtained after 12 h IPTG induction. Each lane contained 15 μg of protein. M: Molecular weight standards (New England Biolabs: P7708S). Lane 1: Cell extract after DNase treatment. Lane 2: Cell extract after the 50 kDa tangential ultrafiltration step. Lane 3: ScNAL after HisTrap column step (purified protein is about 35 kDa).

purification, because 86% recovery was achieved (Table 1), which is 2−4-fold higher than the recovery described for LpNAL, whose purification was performed in three steps (42.3% recovery),[9] and for *Clostridium perfringens* aldolase, which needed five purification steps (21% recovery).[19]

ScNAL, thus obtained, showed a specific activity of 12.7 U/mg for the hydrolysis of Neu5Ac at 37 °C and pH 7.0 (Table 1). Up to 403 mg of Ni^{2+}column-purified ScNAL could be obtained from 1 L of *E. coli* Rosetta (DE3)pLys culture, which is a 2-fold increase as compared to the best expression level reported for LpNAL (215 mg L^{-1}) [9] and 4-fold higher than for other NALs.[8] The molecular weight of purified protein was determined by gel filtration (126.40 kDa), by HPLC/MS/ESI (37.33 kDa) (data not shown), and by 12% SDS-PAGE (35 kDa) (Figure 2, lane 3), confirming the homotetrameric nature of ScNAL.

Biochemical Characterization of Recombinant ScNAL. The activity of ScNAL was pH-dependent and was active over a broad pH range in both synthetic (Figure 3a, ■) and hydrolytic directions (Figure 3a, ●). The optimum pH of the enzyme in both directions was around pH 7, which is similar to the values described for other NALs, such as those from *E. coli* K1 and C600,[20,21] the native and the recombinant NAL from *C. perfringens*,[22] the recombinant NAL from *P. multocida*,[23] and the recombinant NAL from *L. plantarum* WCFS1.[9]

ScNAL showed 50% activity in the synthetic direction above pH 9.0 when glycine was used as a buffer (Figure 3a). In addition, the enzyme was quite stable at neutral pH's (pH 6.0−7.0), and also at basic pH's, where it maintained around 60% residual synthetic activity after 2 h incubation at pH 9, and 90% residual synthetic activity after 2 h incubation at pH 8 (Figure 3b). ScNAL was less stable at such basic pH's as compared to other previously studied NALs, such as EcNAL, which maintains 70% of its activity after 8 h at pH 10.5,[24] or

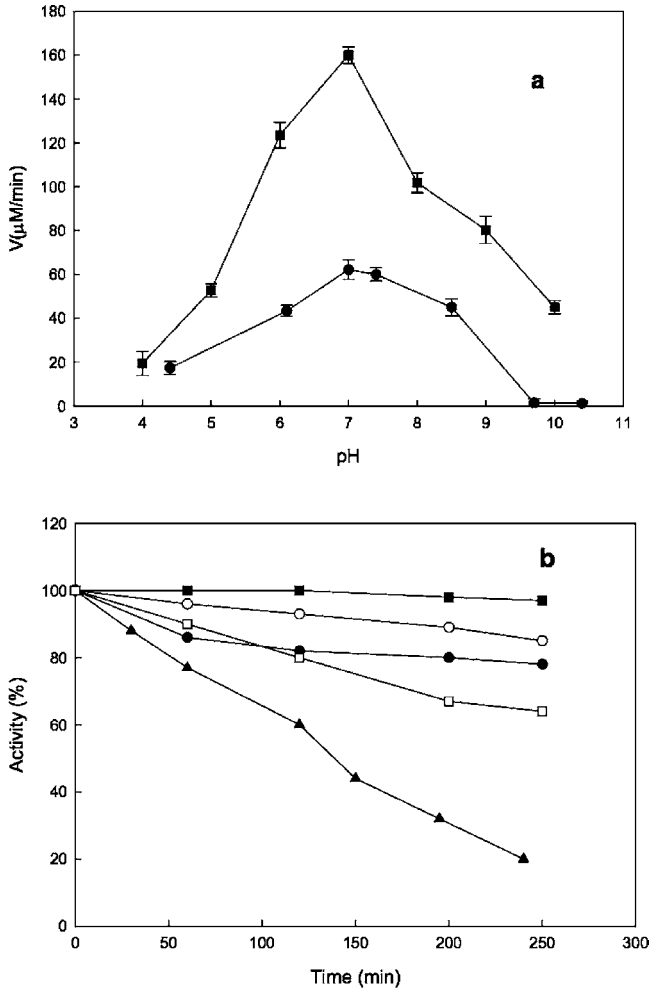

Figure 3. Effect of pH on ScNAL activity. (a) pH profile for Neu5Ac synthesis (■) and hydrolysis (●) determined by HPLC. Assay conditions at 37 °C were 100 mM *N*-acetyl-D-manosamine, 30 mM pyruvate, and 20 μg of enzyme for the synthetic assay, and 10 mM Neu5Ac and 4 μg of enzyme for the hydrolytic assay. The buffers (20 mM) used were sodium acetate pH 4.5−5, sodium phosphate pH 6−7, Tris-HCl pH 8.5, and glycine pH 9−10.5. Independent experiments were conducted with a minimum of three replicates per condition. (b) pH−stability profiles of the purified ScNAL at 37 °C. The enzyme was incubated for different periods of time at pH 5 (□), pH 6 (○), pH 7 (■), pH 8 (●), and pH 9 (▲), and the activity was measured by HPLC under the standard reaction conditions at pH 7, using ManNAc and pyruvate as substrates.

Table 1. Purification of Recombinant ScNAL

purification step	volume (mL)	total activity[a] (U)	total protein (mg)	specific activity (U/mg)	purification (*x*-fold)	yield (%)
crude extract[b]	540	6137	3624	1.7	1	100
50 kDa tangential ultrafiltration	244	6041	2049	2.9	1.7	98
HiPrep IMAC	234	5116	403	12.7	7.5	86

[a]The activity was assayed in the standard spectophotometric reaction medium. [b]Crude extract represents the volume obtained after lysis, centrifugation of cell debris, and DNase treatment, corresponding to a 1 L culture broth. See Materials and Methods for details.

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Lactobacillus plantarum NAL, which maintains around 80% residual synthetic activity after 15 days of incubation at pH 9.[9]

The optimal temperature of ScNAL was measured by HPLC and was found to be 50 °C for synthetic activity (Figure 4a, ■)

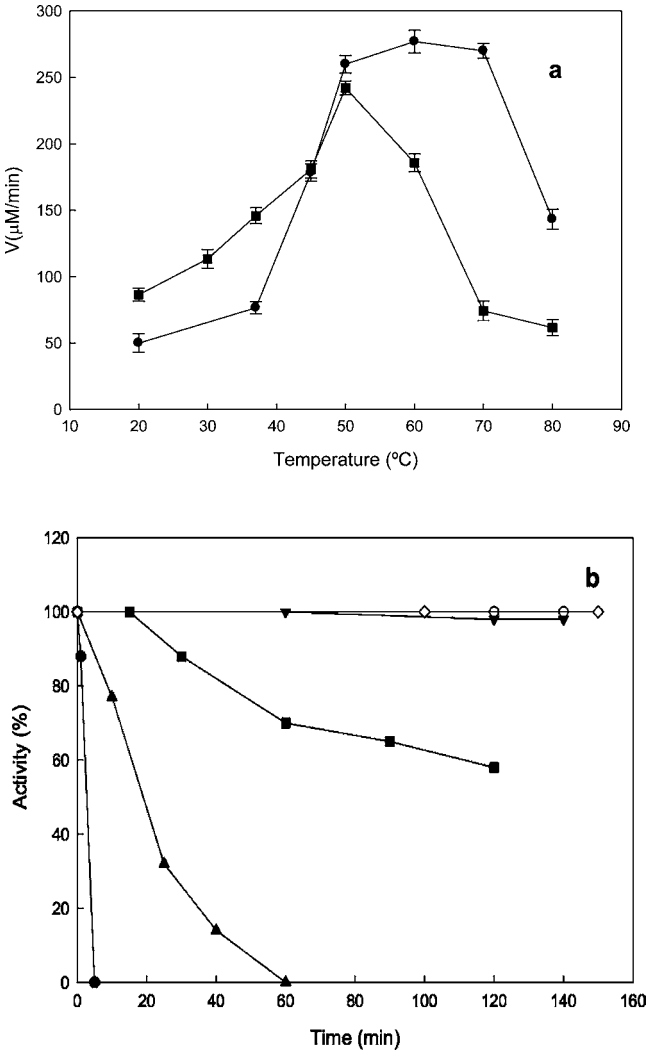

Figure 4. Effect of temperature on ScNAL activity. (a) Temperature profile for Neu5Ac synthesis (■) and hydrolysis (●). Conditions were the same as in Figure 3, but at different temperatures. Independent experiments were conducted with a minimum of three replicates per condition. (b) Temperature stability profiles of the purified ScNAL at pH 7.0. The enzyme was incubated for different periods of time at 20 °C (◇), 37 °C (○), 50 °C (▼), 60 °C (■), 70 °C (▲), and 80 °C (●), and then the activity was measured spectrophotometrically, under the standard reaction conditions at 37 °C using Neu5Ac as the substrate.

and of 60−70 °C for the hydrolytic reaction (Figure 4a, ●), which is somewhat lower than that described for EcNAL (80 °C) [20] and similar to LpNAL (70 °C).[9] The different optimal temperatures between synthetic and hydrolytic reactions are the consequence of the unfavorable equilibrium constant of the synthetic reaction as the temperature increases,[25] which affects the kinetic constants as described in the Haldane equation.[26] Both temperatures are far from the optimal growth temperature of *Staphylococcus carnosus* TM 300, 30−37 °C, the range in which the kinetic parameters of NAL are usually determined.[20,23,9] Thermal stability was also determined in a temperature range of 20−80 °C (Figure 4b). ScNAL was seen to be most stable at temperatures between 60 and 70 °C, but much less stable above 80 °C.

This thermal stability was further confirmed by a thermal melt assay. A T_m value of 62 ± 0.2 °C was obtained for ScNAL in Milli-Q water (Figure 5, ◇). The presence of a buffer

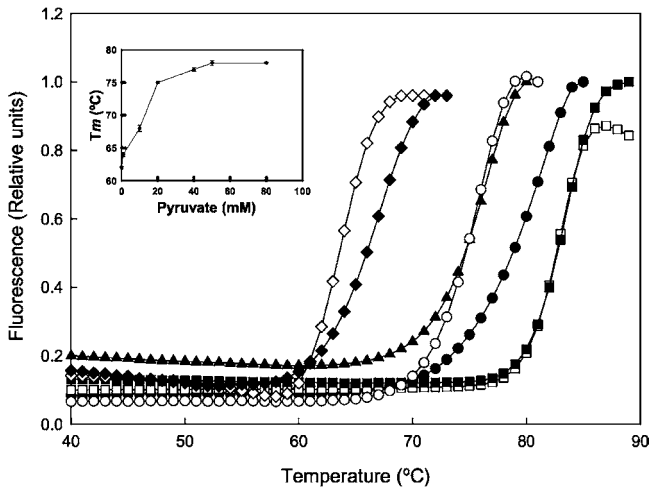

Figure 5. Study of thermal stability of ScNAL. Melting temperature curves of purified enzyme (1 μg) were used in Milli-Q water (◇), in the presence of 20 mM buffer pH 7 (◆) and in the presence of additives, such as 1 M hydroxy-ectoine (▲), 1 M ammonium sulfate (■), 0.4 M ammonium sulfate (□), 20 mM pyruvate (○), and 80 mM pyruvate (●). Assays were performed in real time PCR equipment with 10X Sypro Orange. Inset: Effect of pyruvate concentration (0−80 mM) on melting temperatures of ScNAL.

solution (100 mM sodium phosphate pH 7.0) stabilized the enzyme, raising the T_m value to 66 ± 0.2 °C (Figure 5, ◆). This protective effect was more evident with protein stabilizers. Hydroxy-ectoine (1 M) and ammonium sulfate (0.4 and 1 M) raised the T_m of ScNAL to 76 ± 0.3, 80 ± 0.1, and 82 ± 0.2 °C, respectively (Figure 5). In addition, ScNAL showed great thermal stability between pH 5 and 7.8, with the T_m ranging from 60 to 66 °C, whereas T_m fell drastically to only 40 °C above pH 8.0 (data not shown). These data were in agreement with the optimal pH obtained (Figure 3a). These changes are similar to those studied previously for other proteins, such as LpNAL[9] or anthrax protein.[27]

To study the possible structural changes induced by substrates (ManNAc and pyruvate) and related compounds (D-mannose, glyoxylic acid, and 2-butanone), the T_m values of ScNAL in their presence were calculated for the first time. Sodium pyruvate (0−80 mM) was the only compound affecting T_m, which increased from 62 ± 0.2 to 78 ± 0.1 °C at 80 mM pyruvate (Figure 5 inset; see also Figure 5, ●). These results suggest a direct interaction between pyruvate and the enzyme via a Schiff base intermediate, as has been described in crystals of EcNAL with hydroxypyruvate.[28]

Kinetic Parameters. The kinetic constants were determined in both cleavage and synthetic directions (Table 2). The K_M for Neu5Ac cleavage (2 mM) was lower than the values reported for *E. coli* NAL (2.5−3.8 mM),[20] *P. multocida* (4.9 mM),[23] and *C. perfringens* (2.8−3.2 mM)[22] and similar to that of *L. plantarum* (1.8 mM).[9] This value, together with the k_{cat}/K_M value obtained (2 mM^{-1} s^{-1}), point to the similar catalytic activity of this new recombinant ScNAL for hydrolysis to that

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Table 2. Kinetic Parameters of ScNAL for Neu5Ac Cleavage and Synthesis[a]

activities:	cleavage	Neu5Ac synthesis	
substrates:	Neu5ac	ManNAc	pyruvate
K_M (mM)	2.0 ± 0.3	149.0 ± 0.1	14.0 ± 0.2
k_{cat} (s^{-1})	4.0 ± 0.1	4.5 ± 0.4	2.9 ± 0.3
k_{cat}/K_M (mM^{-1} s^{-1})	2	0.03	0.21

[a]The activity was assayed by HPLC in each standard reaction medium for cleavage and synthesis, respectively (see Materials and Methods for details).

of other previously reported NALs with the same substrate (3.1−4 mM^{-1} s^{-1}).[9]

On the synthetic side, ScNAL showed K_M values of 149 and 14 mM for ManNAc and pyruvate, respectively. These values were also lower than those described for *E. coli* NAL (180 and 22 mM), *P. multocida* NAL (220 and 23 mM),[23] and *L. plantarum* NAL (160 and 19.9 mM).[9] As occurs in Neu5Ac hydrolysis, ScNAL was also efficient for synthesizing Neu5Ac, with k_{cat}/K_M values of 0.03 mM^{-1} s^{-1} for ManNAc and 0.2 mM^{-1} s^{-1} for pyruvate. These values were similar to those previously reported for other NALs.[9]

Neu5Ac Synthesis. To investigate the potential of ScNAL for the production of the Neu5Ac in aqueous solution, the condensation of ManNAc and pyruvate by ScNAL was studied. In this enzymatic reaction, an excess of pyruvate over ManNAc is generally used to achieve high yields of Neu5Ac, because the equilibrium tends toward ManNAc and pyruvate (K_{eq}, 28.7 M^{-1} at 25 °C).[29] For this reason, the reaction was carried out at 25 °C with an almost 2-fold molar excess of pyruvate over ManNAc (1.05 vs 0.55 M). Under these conditions, the mass balance was complete, indicating that no side reactions or decompositions took place. A high conversion (94%) into Neu5Ac was obtained at all of the enzyme concentrations used (3−12 U/mL) (Figure 6). This high conversion was achieved in only 180 min at the highest ScNAL concentration. Such results are similar to the best conversion described (94.4%) using commercial *E. coli* NAL,[13] and higher than those obtained with recombinant *E. coli* C600 NAL,[29] using an 8-fold excess of pyruvate over ManNAc. In addition, this conversion was independent of the feeding strategy used and was the same whether pyruvate was added once or fed three times with 0.35 M, after reaching the equilibrium points at 4, 7, and 20 h, respectively (data not shown).

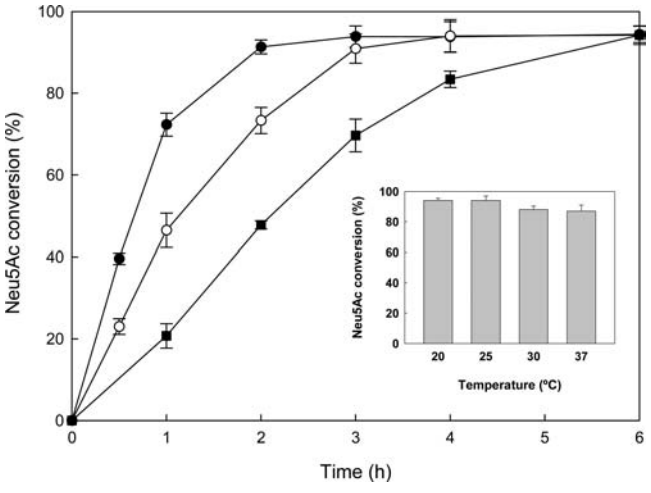

Figure 6. Time course of Neu5Ac synthesis from ManNAc and pyruvate using ScNAL as biocatalyst. The experiments were carried out at 25 °C with 0.55 M ManNAc, 1.05 M pyruvate, and different ScNAL concentrations: 3 (■), 6 (○), and 12 U/mL (●). Inset: Effect of temperature on Neu5Ac production. Conditions are the same as above (12 U/mL), except for temperature. Independent experiments were conducted with a minimum of three replicates per condition.

Apart from the substrate ratio, temperature was also seen to be important due to its dramatic effect on the equilibrium, as described by Lee et al.,[25] who reported that the conversion yield of Neu5Ac increased from 40% at 50 °C to 70% at 20 °C with *E. coli* NAL. Under the conditions used for synthesis with ScNAL, the final maximal conversion achieved was higher (~90%), although it decreased at temperatures above 25 °C, as shown in Figure 6 (inset). Hence, the fastest production of Neu5Ac occurred at a 2:1 pyruvate/ManNAc ratio (1.05 M/0.55 M) and low temperature (25 °C) with 12 U/mL ScNAL.

In conclusion, this study showed that it is possible to use *Staphylococcus carnosus* NAL to synthesize Neu5Ac, in the same time and with the same conversion yield, and using the same units as reported for the best industrial biocatalyst. In addition, the aldolase was obtained from a gene of a GRAS microorganism rather than from a pathogenic microorganism, and there were no problems with the scaling up, due to the high expression and activity of the ScNAL. The above advantages, together with its easy purification and the high enzyme recovery obtained, which lower biocatalysts costs, the simple working conditions used (2:1 molar ratio pyruvate/ManNAc, pH 7.0, no buffer, 25 °C), the short conversion time (3 h at 12 U/mL ScNAL), and the direct crystallization of Neu5Ac from the reaction solutions using glacial acetic acid,[30] make ScNAL a promising biocatalyst for obtaining Neu5Ac for fortified foods, including infant formulas.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

Neu5Ac, *N*-acetylneuraminic acid; NAL, *N*-acetylneuraminate lyases; ManNAc, *N*-acetyl-D-mannosamine; ScNAL, NAL from *Staphylococcus carnosus*; DHA, docohexanoic acid; BLAST, Basic Local Alignment Search Tool; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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