UNIVERSIDAD DE MURCIA

FACULTAD DE BIOLOGÍA

“Producción y caracterización de biocatalizadores implicados en la obtención de ácido siálico y compuestos relacionados”

María Inmaculada García García

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"Producción y caracterización de biocatalizadores implicados en la obtención de ácido siálico y compuestos relacionados"

"Production and characterization of biocatalysts involved in obtaining sialic acid and related compounds"

Memoria presentada para aspirar al título de Doctor por la Universidad de Murcia.

María Inmaculada García García

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AUTORIZAN

La presentación de la Tesis Doctoral titulada "**Producción y caracterización de biocatalizadores implicados en la obtención de ácido siálico y compuestos relacionados**", realizada por **María Inmaculada García García**, bajo nuestra dirección y supervisión, en el Departamento de Bioquímica y Biología molecular-A, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

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  (* Ambos autores han contribuido de igual forma al trabajo)

- **García-García M.I**., Sola-Carvajal A., Sánchez-Carrón G., García-Carmona F., Sánchez-Ferrer A. 2011. New stabilized FastPrep-CLEAs for sialic acid synthesis. Bioresource Technol. 102 (10), 6186-6191.
- **García-García M.I**., Sola-Carvajal A., García-Carmona F., Sánchez-Ferrer A. 2012. Characterization of a novel N-acetylneuraminate lyase from *Staphylococcus carnosus TM300* and its application to N-acetylneuraminic acid production. J. Agric. Food Chem. dx.doi.org/10.1021/jf3014102 (Aceptado)
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- **García-García M.I**., Sánchez-Ferrer A., García-Carmona F. 2012. Molecular charcaterization of a new N-acetylneuraminate synthase from *Idiomarina loihiensis*. (Enviado)

“Soy de las que piensan que la ciencia tiene una gran belleza. Un científico en su laboratorio no es sólo un técnico: es también un niño colocado ante fenómenos naturales que le impresionan como un cuento de hadas. “

Marie Curie

A mis padres

Abbreviations

AS-CLEAs	Ammoniun sulphate CLEAs
ATP	Adenosine 5′-triphosphate
BoAGE	Recombinat N-acetyl-D-glucosamine-2-epimerase from *Bacteroides ovatus* ATTC 8483
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
BSA-CLEAs	Bovine serum albumin-CLEAs
CFR	Code of Federal Regulations
Chlor	Choramphenicol
CLEAs	Cross-linking enzyme aggregates
CLECs	Cross-linked enzyme crystals
CLIBs	Crosslinked inclusion bodies
CpNAL	*Clostridium perfringes* recombinant N-acetylneuraminate lyase
Da	Dalton
EcNAL	*E. coli* recombinant N-acetylneuraminate lyase
EDTA	Ethylenediaminetetraacetic acid
ELSD	Evaporative light scattering detector
FP-CLEAs	FastPrep-CLEAs
FPCL	Fast proteins liquid chromatography
FspNAMDH	Recombinant N-acetyl-D-mannosamine deydrogenase from *Flavobacterium* sp.
g	Constant *gravity*
GlcANc	N-acetylglucosamine
GlcANc-6P	N-acetylglucosamine 6-phosphate
GRAS	Generally Recognized as Safe
GRAVY	Grand average of hydropathicity

HiNAL	Recombinant N-acetylneuraminate lyase from *Haemophilus influenza*
HMO	Human milk oligosaccharides
HPLC	High performance liquid chromatography
IiNAS	Recombinant N-acetylneuraminate synthase from *Idiomarina loihiensis* L2TR
IPTG	Isopropil-β-D-1thigalactopyranoside
Kan	Kanamycin
KDN	2-keto-3-deoxy-d-glycero-D-galacto 2-nonulosolic acid
KDO	3-deoxy-D-manno-octulosonic acid
k_{cat}	Catalytic constant
K_M	Michaelis-Menten constant
LB	Luria bertani medium
LDH	Lactate dehydrogenase
LaNAL	Recombinant N-acetylneuraminate lyase from *Lactobacillus antri*
LpNAL	Recombinant N-acetylneuraminate lyase from *Lactobacillus plantarum* WCFS1
LsNAL	Recombinant N-acetylneuraminate lyase from *Lactobacillus sakei* 23k
ManNAc	N-acetylmannosamine
NAL	N-acetylneuraminate lyase
NAS	N-acetylneuraminate synthase
$NCAM_S$	Neuronal cell adhesion molecules
NCBI	National Center for Biotechnology Information
Neu5AC	N-acetylneuraminic acid/ sialic acid
NJ	Neighbor joining
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
SB-CLEAs	Sodium borohydride CLEAs

ScNAL	Recombinant N-acetylneuraminate lyase from *Staphylococcus carnosus* TM300
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-Polyacrilamide gel electrophoresis
SEM	Scanning electron microscope
TB	Terrific Broth
TEMED	N,N,N´,N´,-Tetramethylethylenediamine

"Esta tesis doctoral está sometida a procesos de protección o transferencia de tecnologíao de conocimiento, por lo que los siguientes contenidos están inhibidos en la publicación en los repositorios institucionales.

Autorizado por la Comisión General de Doctorado de la Universidad de Murcia con fecha 21 de Febrero de 2013.

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INTRODUCTION

Materials and Methods

I.General Introduction

1. INTRODUCTION

Sustainable development is a task all we must all contribute to. The chemical industry, for its part, has committed itself to sustainable development to meet the needs of the present without compromising future generations. There are several approaches to making production processes in the chemical industry more sustainable. One approach is the application of biotechnology in the production of new products with to replace old production processes or single reaction steps (Liese, 2002). In this respect, biocatalytic processes have several advantages compared with classical chemical processes, since, due to moderate reaction conditions and high selectivity the energy demand involved and amount of waste produced are relatively low. However, despite these advantages biocatalytic processes are often not competitive compared with classical processes, that have been developed and optimized over decades, while biotechnology and its application at industrial scale is comparatively young. Therefore biotechnology has a lot of catching up to do when it comes to the enhancement of catalysts, process engineering and the development of downstream processing methods to recover the products from aqueous solutions often in low concentrations.

An interesting group of biocatalytic reactions are the diastereo- and enantio-selective aldol condensations of an aldehyde and a ketone or two aldehydes catalysed by aldolases (aldehyde-lyases, E.C. 4.1.2.x). The products, thus obtained are valuable building blocks in the emerging field of glycobiotechnology (Faber, 2004, Huang *et al*. 2007, Yu *et al*. 2006).

Unfortunately, large-scale production is often not economically viable due to an unfavorable equilibrium of the catalyzed reaction. Therefore the application of

new tools and methods for process optimization, such as modeling (to find the best operating conditions) or the concurrent removal of product during the reaction (to shift the equilibrium) are of great interest in these reactions. Some of the most interesting enzymes are N-acetylneuraminic acid aldolase (E.C. 4.1.3.3) and N-acetylneuraminte synthase (E.C. 2.5.1.56) because they catalyses the synthesis of N-acetylneuraminic acid, an amino sugar often linked to oligosaccharides in the terminal position in mammalian cells. N-acetylneuraminc acid and its derivatives are interesting compounds in various therapeutical applications.

1.1 SIALIC ACID

Sialic acid is a generic term to indicate a wide family of more than 50 structurally distinct sugars with α-keto acids on a nine-carbon backbone (Angata and Varki, 2002; Vimr *et al*., 2004). The most common form of silaic acid contains the N-acetyl group in C5 position to give N-acetylneuraminc acid (Neu5Ac) while a hydroxyl group in the same position produces 2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid (KDN), and includes pseudoaminic acid, 2-keto-3-deoxy-D-manno-octulosonic acid (KDO) and 3-deoxy-D-arabino-heptulosonate -7-phosphate (DAH-7-P). The N-acetyl group itself can be hydroxylated to produce N-glycolylneuraminc acid (NeuNGc) (Figure, 1.1). Over 60 derivates of Neu5Ac and KDN are known, which differ in some modification at C-4, C-7, C-8 and C-9 with acetate, lactate, sulfate, phosphate and methyl (Tao *et al*., 2009)

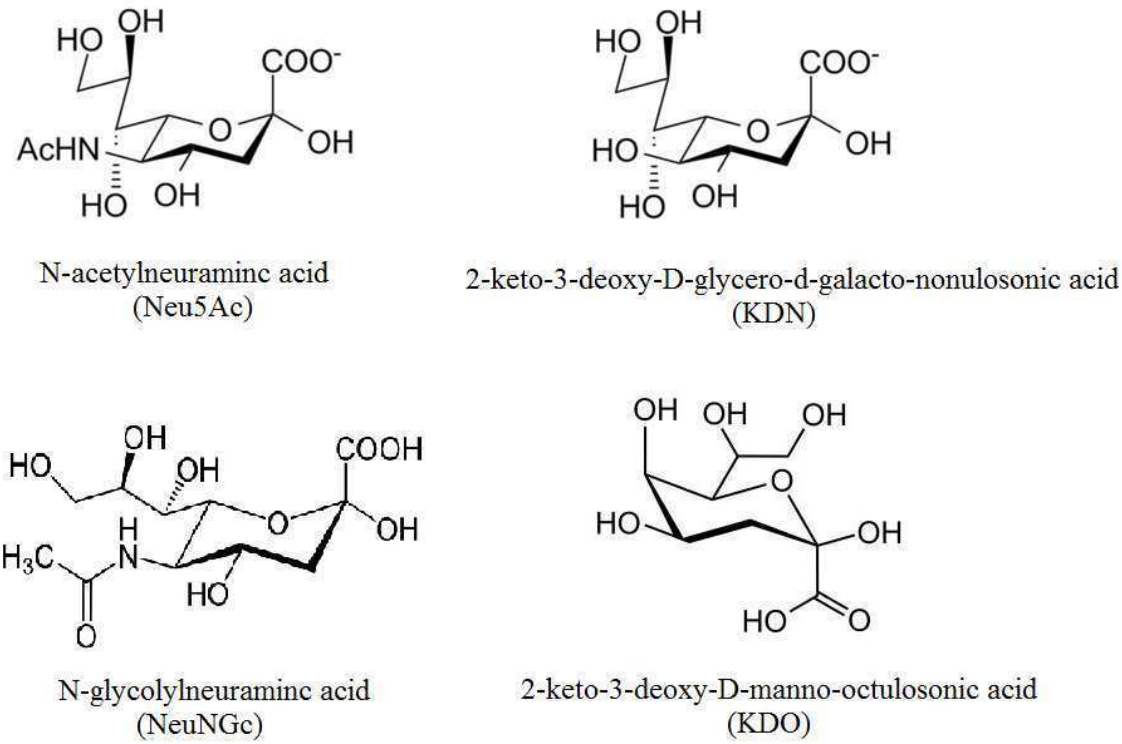


Figure 1.1: *Sialic acid and some derivates.*

These sugars play a vital role in a variety of biological functions and physiological and pathological processes, such as the development, recognition, cell adhesion, microbial binding leading to infections, the regulation of immune response, and the progression and spread of human malignancies (Chen and Varki 2010; Schauer 2000; Traving and Schauer 1998). Their prevalence is widespread as they are observed in viruses, mammalian cells, and certain microbial organisms (Gunawan *et al.*, 2005)

1.1.1 History of sialic acid

The story begins in the mid 1930s when this sugar was isolated from submaxillary mucin by Gunnar Blix in 1936, leading to the name “sialic acid” for this saliva-derived acidic compound. Shortly thereafter, by the early 1940s, Ernst Klenk independently isolated acidic glycosphingolipids (GSLs) (Klenk, 1941), which were

named gangliosides because they were abundant in brain gray matter and ganglial cells. Klenk identified sphingosine, fatty acid, hexoses, as well as a substance that gave a purple color with Bial's reagent that he called neuraminic acid, as constituents of the brain-derived glycolipids. Klenk's neuraminic acid was later found to be the same compound isolated from saliva by Blix and the nomenclature was clarified in 1957 (Blix *et al*., 1957) with the name "sialic acid" now collectively referring to a family of over 50 naturally occurring sugars (Angata and Varki, 2002), as well as a growing number of synthetic sugars achieved through metabolic substrate-based sialic acid engineering methods (Keppler *et al*., 2001; Yarema 2001).

1.1.2 Properties and biological roles

Sialic acids are mainly found in certain species of animals and bacteria, and with the sole exception of DAH-7-P, as components of the cell surface polysaccharides or glycoconjugates. Among animals, they are confined to the deuterostome lineage (vertebrate, ascidians and echinoderms) and among bacteria; it appears in the pathogenic bacteria that infect these animals.

Neu5Ac and its derivatives have numerous important physiological functions, which are closely related to the structural diversity of these molecules (Figure 1.2) (Schauer, 2000).

The major functions can be divided into the following categories:

1) Molecular and cellular attraction and repulsion and in the binding and transportation of positively charged molecules (Tao et al., 2009)

2) Maintaining the molecular conformation of enzymes and proteins on the cell surface (Tao *et al*., 2009).

3) Cellular and molecular recognition processes due to their structural diversity (Crocker *et al*., 1998).

4) Anti-recognition by masking antigens, receptors, and other recognition sites on molecules and cells (Schauer *et al*., 1984).

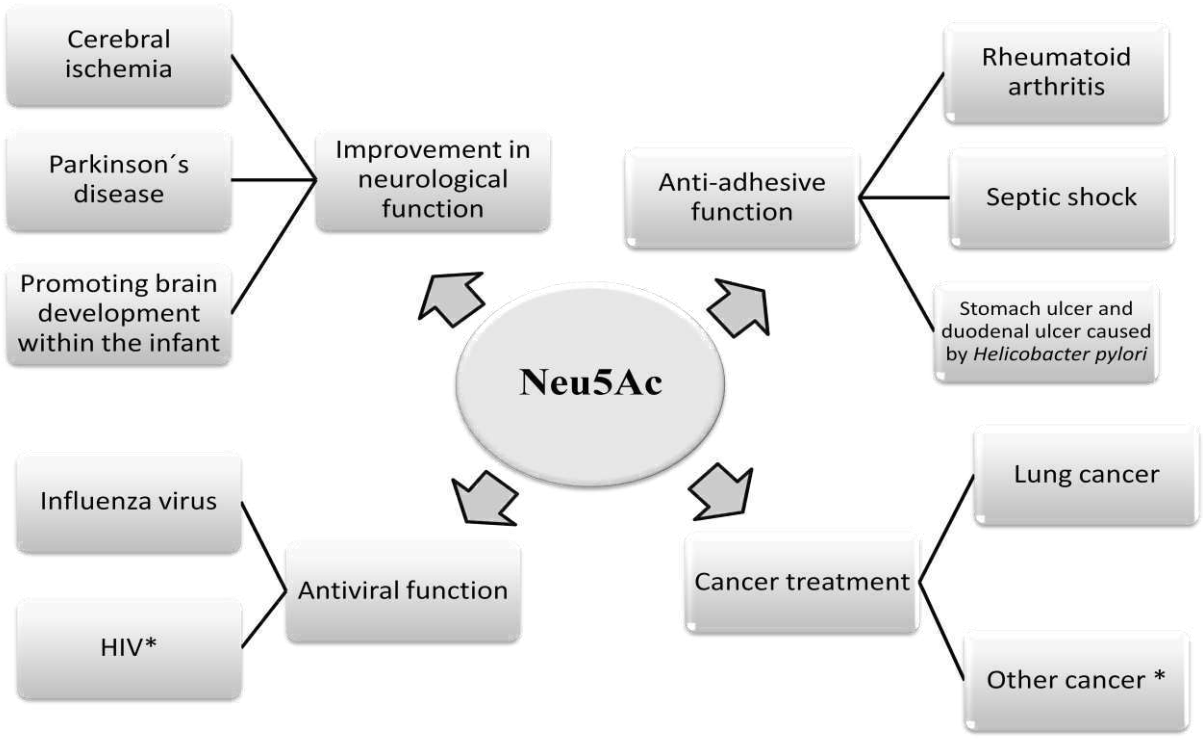


Figure 1.2: *The current function and application of Neu5Ac. (*) Represents potential uses.*

Sialic acid is known to be a key monosaccharide unit in brain gangliosides and glycoproteins, including poly-N-acetylneuraminic acid glycotope on neural cell

adhesion molecules (NCAM), which function in the CNS to regulate cell migration, neurite outgrowth, axon elongation, and synaptic formation and plasticity (Kiss and Rougon, 1997; Rutishauser and Landmesser, 1996). The adhesion properties of NCAMs are mainly due to hemophilic binding. During the early stages of neural cell develompment, surface-dipalayed NCAMs are glycosylated with polysialic acid (PSA), a homopolymer of up to 200 α-(2 →8)-linked sialic acids. The high surface charge and hydration brought about by sialylation inhibits the hemophilic binding of NCAMs. In later stages, NCAMs are desialytated and regain their full adhesion fuction (Cunningham *et al*., 1983; Kleene & Schachner, 2004).

1.2 BIOTECHNOLOGICAL AND MEDICAL INTEREST OF SIALIC ACID

1.2.1 Antiviral intermediate

Influenza, commonly referred to as the flu, is an infectious disease caused by RNA viruses of the family Orthomyxoviridae (the influenza viruses), that affects birds and mammals (Kilbourne, 2006; Oxford, 2000). Influenza spreads around the world in seasonal epidemics, resulting in the deaths of between 250,000 and 500,000 people every year. Three influenza pandemics occurred in the 20th century, in 1918 (Spanish Flu), 1957 (Asia Flu) and 1968 (Hong-Kong Flu) and killed tens of millions of people, each of these pandemics being caused by the appearance of a new strain of the virus in humans. An avian strain named H5N1 raised the concern of a new influenza pandemic, after it emerged in Asia in the 1990s, but it has not evolved to a form that spreads easily between people.

Neu5Ac is also used to synthesize antiviral agents, and this has resulted in an increased demand for Neu5Ac worldwide. Sialidase plays an important role in

microbial infection (for example, in viral infection) and dissemination processes. Derivatives and structural analogs of Neu5Ac can effectively inhibit sialidase and can therefore be used as antiviral drugs for preventing virus infection and release. Anti-influenza virus drugs such as zanamivir (4-guanidino-Neu5Ac2) and oseltamivir have a strong specific inhibitory effect on sialidase and have a broad antiviral spectrum (Figure 1.3). These drugs can effectively inhibit the spread of the influenza virus throughout the body. In 1999, the drugs were approved for the treatment of A- and B-type influenza by the US Food and Drug Administration (Dreitlein *et al*., 2001). Based on the antiviral mechanism of sialidase inhibitors, structural analogs can be obtained by simple alteration of some of the enzyme binding sites (Moscona, 2005). Therefore, even if virus mutations do occur, antiinfluenza virus drugs can still be designed and synthesized in a timely manner by combining rational design and combinatorial chemistry. Recently, it has been reported that zanamivir is useful in preventing some oseltamivir-resistant influenza A H1N1 influenza viruses (Soundararajan *et al*., 2009). This type of drug played an important role in controlling the recent global H1N1 influenza virus pandemic, and the enormous potential of this type of anti-flu drugs has been clearly demonstrated in the control of influenza (Kawai *et al*., 2009; Soundararajan *et al*., 2009; von Itzstein, 2007).

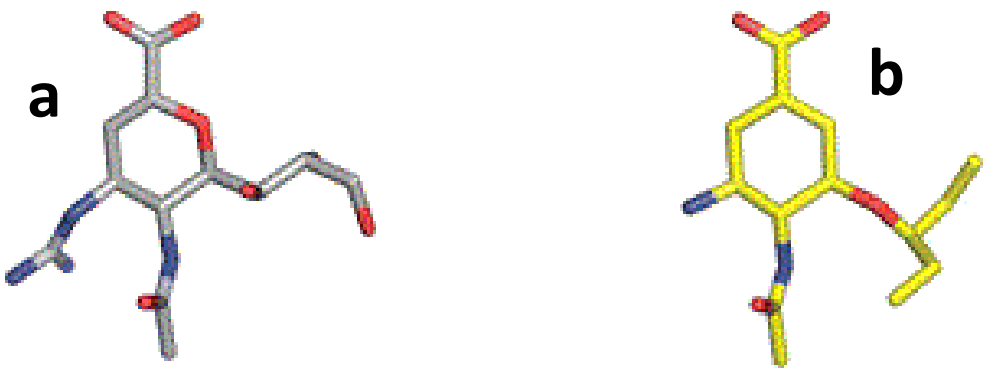


Figure 1.3. *Chemical structure of sialic acid derivates*. a) Zanamivir. b) Oseltamivir.

1.2.2 Enrichment of infant formula

The rapid growth of infant brains places an exceptionally high demand on the supply of nutrients from the diet, particularly for preterm infants. Any deficit has profound effect on somatic growth and organ structural and functional development, especially the brain. The rapid initial growth of the brain exceeds that of other body tissues. In the 6th month gestation, it comprises 21% of total body weight and 15% at term (Friede, 1989). The brain weight more than doubles during the first 9 postnatal months to reach over 90% of the adult weight by the 6th year. Once this critical period of brain growth has passed, it cannot be restarted. The challenge is accentuated in premature infants, particularly as regards to nutritional support for brain growth.

N-acetylneuraminic acid concentrations in brain cortical tissues are also correlate with evolutionary development in higher animals; the human brain contains twice as much N-acetylneuraminic acid as the chimpanzee brain (Wang *et al*., 1998).

Importantly, the N-acetylneuraminic acid concentration in the frontal cortex of breast-fed infants is higher than the levels of formula-fed infants (Wang *et al*., 2003). In addition, the levels of glanglioside N-acetylneuraminic acid correlated significantly with ganglioside ceramide DHA and total ω-3 fatty acids in breast-fed infants, but not in formula-fed infants. This structural and functional link between docohexanoic acid (DHA) and Neu5Ac may act together to benefit early development and cognition (Wang *et al*., 2003). Taken together, these findings suggest that this increase in N-acetylneuraminic acid in breast-fed infants arises from the exogenous dietary supplementation of Neu5Ac, which is found in human milk but not in infant formulas based on cow milk or protein hydrolysates (Wang *et al*., 2001). Thus, premature infants, whose number has increased due to advances in reproductive technologies and who receive artificial feeding, are likely to be at greater risk of neural deficit.

Some of these natural oligosaccharides, including Neu5Ac, have been used as natraceutical product in milks and food for infants to enhance the presumed usefulness of human milk oligosaccharides, especially for brain growth and development. However, those are still a paucity of information on the ideal carbohydrate structure to use and the efficacy of such supplementation (Wang *et al*., 2009; Wang *et al*., 2001).

1.2.3 Synthesis of Neu5Ac

The high price of Neu5ac (50-100 \$ g^{-1}), which results from its conventional method of production, has hindered its industrial application. There are several strategies for preparing Neu5Ac. This compound can be extracted from natural sources such as colominic acid, egg yolk, and milk (Juneja *et al*., 1991; Koketsu *et*

al., 1992; Shimatani *et al*., 1993). However, the sialic acid content in natural sources is relatively low, as a result of which the separation and purification processes are relatively complex and result in relatively low yields. Therefore, conventional methods are unsuitable for large-scale production. One of the routes for the industrial production of Neu5Ac is chemical synthesis (Furuhata, 2004). For example, this compound can be produced by the condensation of N-acetyl-glucosamine (GlcNAc) and oxaloacetate under alkaline conditions (pH 9–11), followed by decarboxylation (Cornforth *et al*., 1958). It can also be prepared by asymmetric synthesis from D-mannose and nonsugar precursors such as 1,2-cisdihydro-catechol (Danishefsky *et al*., 1988; Deninno 1991; Schmidt *et al*., 1990). However, these chemical methods require laborious repetitive sequential protection and deprotection steps and may lead to the formation of numerous intermediates and isomers resulting in a highly complex and difficult separation process. A third strategy for Neu5Ac preparation involves the use of different types of biocatalytic processes including enzymatic catalysis, whole-cell biocatalysis, and fermentation (Schmid *et al*., 2001; Schoemaker *et al*., 2003).

1.2.4 Synthesis of KDN

KDN (2-keto-3-deoxy-D-glycero-D-galacto-nonopyranulosonic acid) is a naturally occurring derivate of sialic acid where the amino group at carbon 5 is replaced by an hydroxyl group. KDN has unique properties and biological functions distinct from those of N-acetylneuraminic acid. For example, KDN ketosidic linkages are resistant to known sialidases from bacteria and animals, which may protect KDN glycoconjugates from bacterial and viral attack (Nadano *et al*., 1986; Yu et al., 1991; Angata *et al*., 1994; Kimura *et al*., 1994; Li *et al*., 1994). KDN is involved in

building block for the synthesis of glycolipids with activities as inmunoregulator and in tumor antimetastasis and against Alzheimer´s disease (Ogura *et al*., 1994). It is these unique properties of KDN that make the development of procedures for its enzymatic synthesis of KDN very interesting. The first enzymatic synthesis of KDN has been described by Augé and Gauthernon (1987), using D-mannose and pyruvate, and one decade later, the enzymatic large-scale production in enzyme membrane reactors was described (Salagnad *et al*., 1997). The same was also, carried out, using fusion proteins of N-acetyl-D-neuraminic acid aldolase (Wang and Lee, 2006).

1.3 SIALIC ACID METABOLISM

1.3.1Catabolism of Neu5Ac

Given the preponderance of sialic acid in complex animals, microorganisms that degrade sialic acid are, by definition, excepted to be closely associate as commensals or pathogens with their animal host. The emergence of microbial sialic acid catabolism is hypothesized to be central to a variety of host-microbe interactions. On the assumption that the above bacteria express an efficient transporter and degradative system for sialic acid dissimilation (Vimr and Troy, 1985a; Vimr and Troy, 1985b) isolated mutants of E. coli K1 which failed to use sialic acid as sole carbon source. The genetic defect in two of these *nan* (for N-acetyl-neuraminte) mutants were subsequently located in genes for sialate transport (*nan*T) and the aldolase (*nan*A)(Vimr and Troy, 1985a). The *nan*AT genes were part of an operon that responded to apparent induction based on sialic acid availability. The results of genetic and physiological studies indicated that exogenous sialic acid is transported by a secondary transporter (NanT) of the

major facilitator superfamily, and degraded intracellulary by NanA, to yield pyruvate and the amino sugar ManNAc (Vimr and Troy, 1985a; Vimr and Troy, 1985b). Upon completion of the *E. coli* K-12 genomic DNA sequencing project (Blattner et al., 1997), the *nan* operon was seen to potentially include two more open reading frames, which were subsequently suggested for phosphorylating ManNAc (*nan*K) (Plumbridge and Vimr, 1999), and then, for epimerizaing the ManNAc-6-P generated to GlcNAc-6-P (*nan*E)(Figure 1.4). Later biochemical analyses confirmed that NanK is an ATP-dependient kinase specific for ManNAc and that NanE is a reversible epimerase (Ringenberg et al., 2003). Since then, a complete nan system has been defined as one that minimally includes orthologues of genes encoding NanA, NanE and NanK.

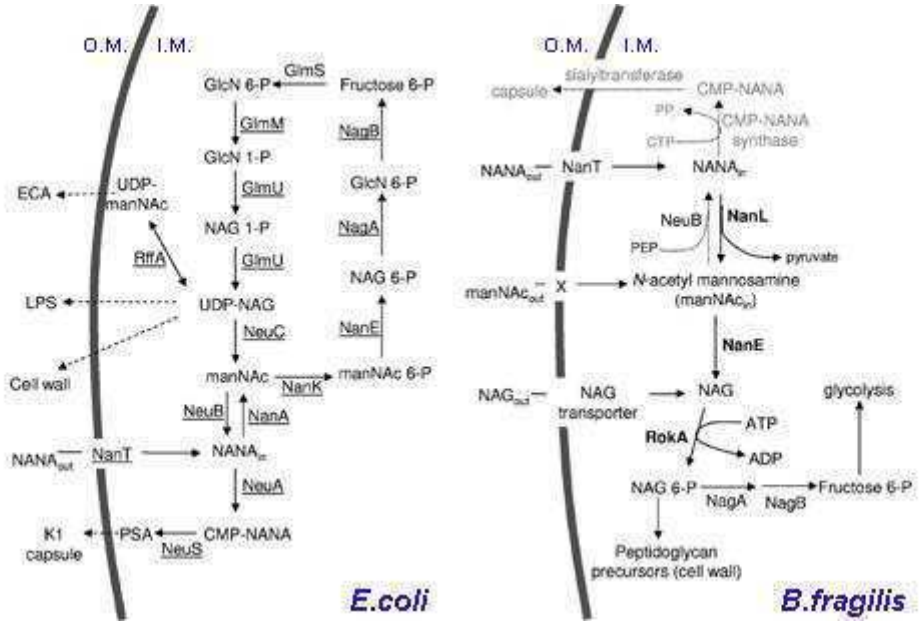


Figure 1.4: *Comparison of Neu5Ac and amino sugar utilization pathways of E. coli and Bacteroides fragilis.* Taken from Brigham *et al* (2009).

Most of the bacteria that degrade sialic aicd probably phosphorylate the ManNAc released by NanA prior to epimerization, as shown for *E. coli* (Figure 1.4)

(Plumbridge and Vimr, 1999, Ringenberg *et al*., 2003). However, some organisms, such as Bacteroides fragilis (Brigham *et al*., 2009), differ significantly from this above described pathaway (Ringenberg *et al*., 2003). Figure 1.4 presents a comparation of these pathaways. The main differences in the Neu5Ac utilization pathways of *E. coli* and *Bacteroides spp*. occur only after Neu5Ac is cleaved by the lyase (NanA), to form ManNAc and Pyruvate. In *E. coli* and many bacteria, including well-studied pathogens, ManNAc is immediately phosphorylated by NanK Kinase to produce ManNAc-6-P. The E. coli NanE epimerase then converts ManNAc-6-P to GlcNAc-6-P, which then enters the amino sugar utilization pathway (Ringenberg *et al*., 2003). In contrast, in *B. fragilis*, the NanE epimerase enzyme converts unphosphorylated ManNAc to GlcNAc, using ATP as a cofactor. In this respect, the *B. fragilis* NanE catalytic function is similar to the mammalian Renin binding protein/N-acetyl-D-glucosamine-2-epimerase (RnBP/AGE). In *B. fragilis* GlcNAc is subsequently phosphorylated by RoKA kinase to yield GlcNAc-6-P, which is further metabolized to Fructose-6-P to enter in the glycolysis pathway (Brigham *et al*., 2009). One important characteristic of the first two enzymes used in the degradation of Neu5Ac (Neu5Ac aldolase and NanE epimerase) is that they also catalyze the reversible reaction, making it possible to use them for enzymatic production of sialic acid.

1.3.2 Anabolism of Neu5Ac

Neu5Ac biosynthesis follows similar routes in bacteria and mammals (Figure 1.5). The first step is catalyzed by the enzyme, UDP-*N*-acetyl-glucosamine 2-epimerase, which inverts the stereochemistry at C2 and hydrolyzes the glycosidic phosphate bond in converting UDP-*N*-acetylglucosamine (UDP-GlcNAc) to *N*-

acetylmannosamine (ManNAc) (Tanner, 2005). The mammalian UDP-GlcNAc 2-epimerase then phosphorylates ManNAc while the bacterial enzyme does not.

In mammals, Neu5Ac-9-phosphate synthase catalyzes the condensation of phosphoenolpyruvate (PEP) with ManNAc-6-P, generating Neu5Ac-9-P. The bacterial enzyme, sialic acid synthase (Neu5Ac synthase), catalyzes a similar condensation of ManNAc and PEP to generate Neu5Ac. The pathways then converge to produce CMP-Neu5Ac. This latter compound is an activated form of Neu5Ac, which is used by CMP-sialyl transferases to synthesize sialic acid-containing oligosaccharides, polysaccharides, and glycoconjugates. Neu5Ac synthase stands out as a drug target because it is unique to the bacterial pathway and it uses a different substrate from its mammalian analogue (Tanner, 2005).

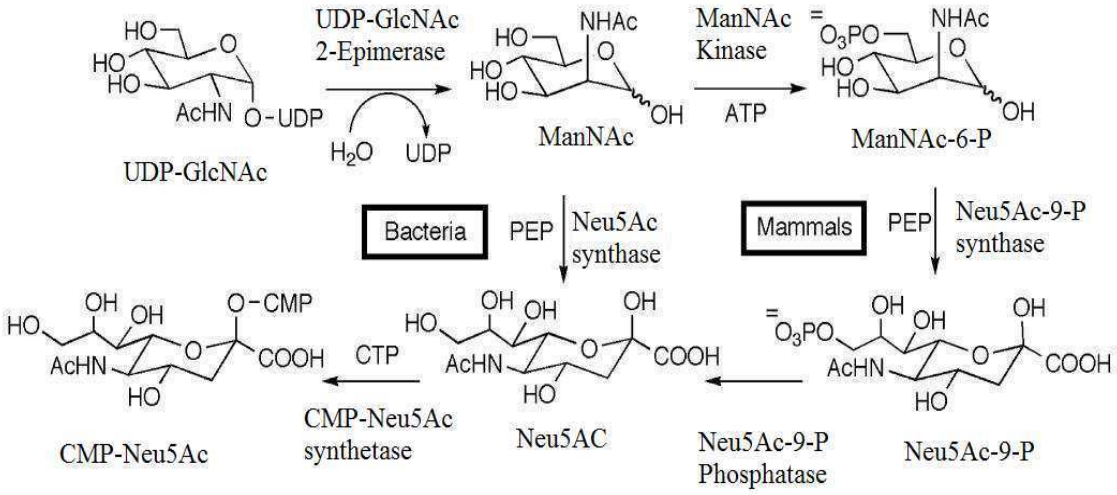


Figure 1.5: *The biosynthesis of CMP-Neu5Ac in bacteria and mammals.*

1.4 ENZYMES INVOLVED IN Neu5Ac SYNTHESIS

1.4.1 N-acetylneuraminate lyase

The enzyme sialic 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 via a Schiff base intermediate, with an equilibrium that favours Neu5Ac cleavage. NAL also catalyzes the reverse aldol condensation reaction and has been used in this way to synthesize sialic acid and some of its derivatives from pyruvate and *N*-acetyl-D-mannosamine (Figure 1.6) (Huang *et al*., 2007; Yu *et al*., 2004). Interest in this aspect of the enzyme´s activity has increased, with growing appreciation of the role of sialic acid in controlling biomolecular interactions, particularly at the cell surface. However, most NALs described to date come from human pathogens: *Escherichia coli* (EcNAL) (Aisaka *et al*., 1986; Ohta *et al*., 1986; Otha *et al*., 1985), *Clostridium perfringes* A99 (Traving *et al*., 1997), *Haemophilus influenzae* (HiNAL) (Lilley *et al*., 1998), *Trichomonas vaginalis* (Meysick *et al*., 1996), *Pasteurella multocida* (Li *et al*., 2008), except for the NAL cloned from the commensal *Lactobacillus plantarum* (LpNAL) (Sánchez-Carrón *et al*., 2011).

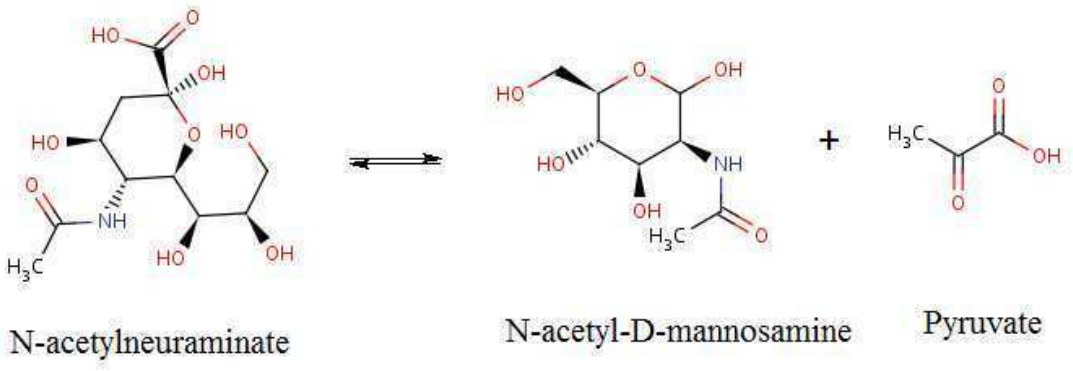


Figure 1.6: *Reaction catalyzed by N-acetylneuraminate lyase.*

1.4.1.1 Structure

The X-ray structure of NAL and some mutants from *E. coli* and *H. influenza* have also been solved (Barbosa *et al*., 2000; Campeotto *et al*., 2010; Campeotto *et al*., 2009; Izard *et al.,* 1994; Joerger *et al*., 2003; Lawrence *et al*., 1997). The enzyme is either a homotretamer (Izard *et al.,* 1994) or a homodimer (Schauer *et al.,* 1999), each monomer of which consists of an $(\alpha/\beta)_8$ barrel decorated at its C-terminus by three α-helices (Figure 1.7). NAL shares this structural framework with other members of the $(\alpha/\beta)_8$ subfamily enzymes (Lawreance *et al*., 1997), such as dihydrodipicolinate synthase (DHDPS) and D-5-keto-4-deoxyglucarate dehydratase (KDGDH), although they catalyse different reactions.

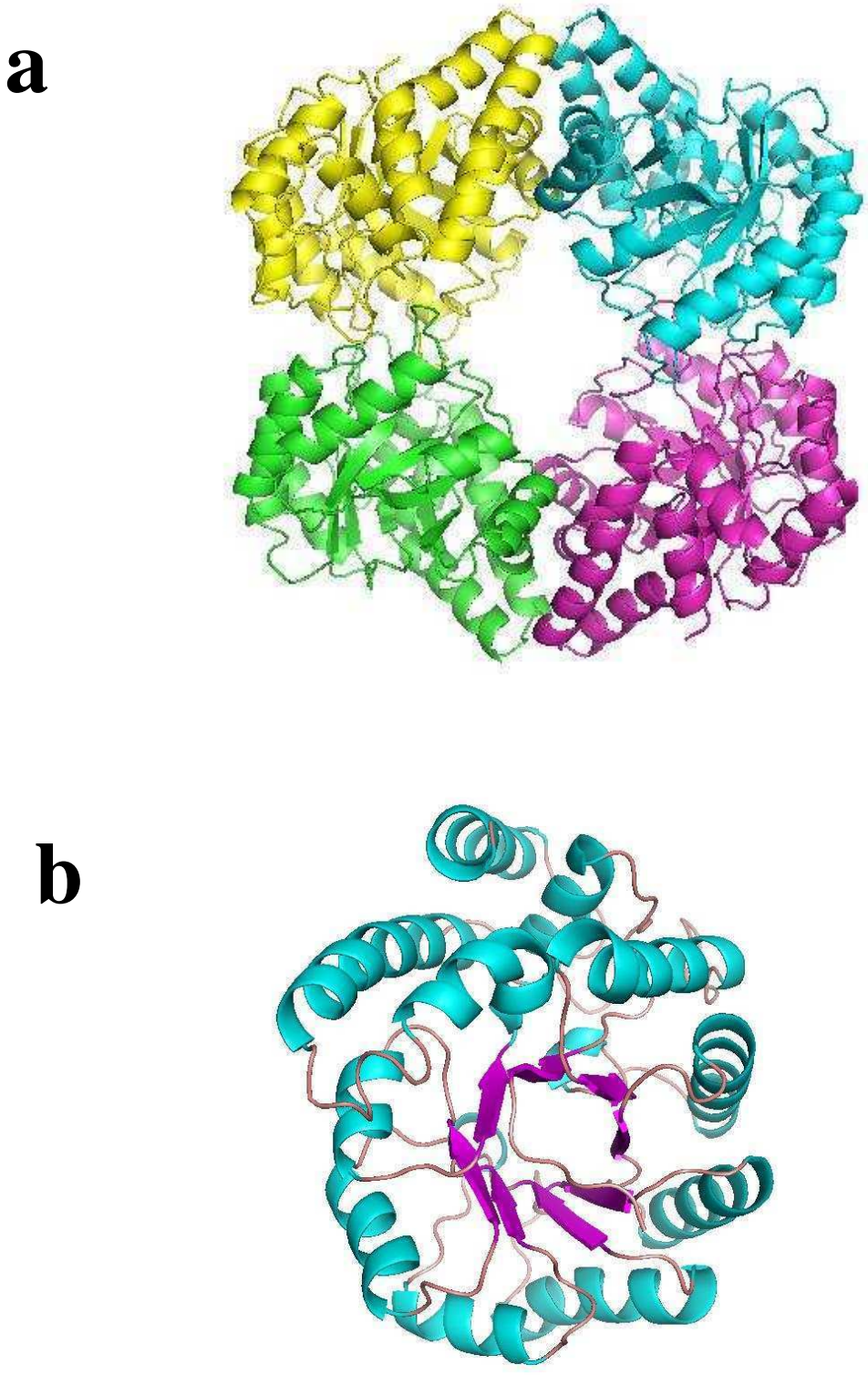


Figure 1.7: *3D view of E. coli NAL* in tetrameric form (a) and as a monomer (b) [pdb code: 1NAL (Izard *et al.*, 1994)].

On the basis of the X-ray structural analysis of covalent complexes of EcNAL (Izard *et al.,* 1994) and HiNAL (Barbosa *et al*., 2000), the existence of a highly conserved structural motif associated with, a Schiff base between a strictly conserved lysine residue and C2 carbon of the common α–keto acid moiety of the substrate has been demostrated. These results are similar to those obtained for *E. coli* DHDPS (Blickling *et al*., 1997). In both, a covalent link is seen between the C2 carbon of pyruvate and the amine nitrogen of the strictly conserved lysine residue (Figura 1.8). In the complexes, the carboxylate moiety of the bound pyruvate is oriented to form hydrogen bonds with the backbone amides of the residues, Ser and Thr, as well as with the hydroxyl of Tyr (Figure 1.8). Serine and threonine form the respective second and third residues of a highly conserved GXXGE motif, the three-dimensional conformation of which can be described as two overlapping type I β–turns (Lawrence *et al*., 1997).

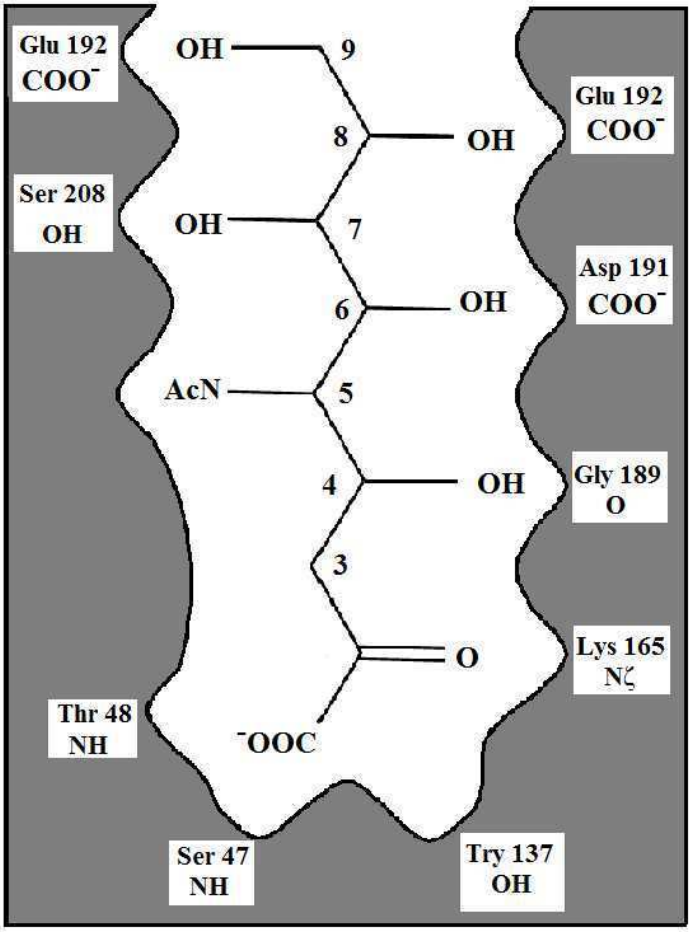


Figure 1.8 *Schematic residues implicated in the catalytic mechanism and their interaction with Neu5Ac.*

1.4.1.2 Phylogeny of N-acetyl neuraminate lyase

Some authors have reported that the gene encoding Nana N-acetyl neuraminate lyase is located within a cluster of genes, known as the Nan Cluster, necessary for the catabolism of sialic acid. The Nan Cluster is formed by N-acetylneuraminate lyase (NanA), epimerase (NanE), and kinase (NanK) (Figure 1.4). The Nan cluster is confined to predominantly pathogenic and commensal bacteria. The cluster was present only in members of the Gamma-Proteobacteria and Fusobacteria among Gramnegative bacteria, and Bacillales, Clostridia, and Lactobacillales among Gram-positive bacteria, as well as *Mycoplasma*. (Almagro *et al*., 2009)

According to recent phylogenetic studies (Sánchez Carrón *et al*., 2011), sialic acid aldolases can be divided into four groups. The first group includes enzymes from Gram-negative genera, *Haemophilus*, *Actinobacillus*, *Pasteurella*, as well as Gram-positive genera, *Lactobacillus*, *Clostridium*, *Staphylococcus* and *Mycoplasma*, and also *Fusobacteria*. Group 2 includes NAL structures from *E. coli*, *Shigella* spp., *Salmonella enterica*, which all are human gastrointestinal pathogens and have high sequence identities (~ 90-100 %). Group 3 is composed of Streptococcus genera, some species of *Lactobacillus* (*L. antri*, *L. sakei*), *Clostridium* (*C. bolteae*, *C. hylemonae*), accompanied by other human gut commensals such as *Ruminococcus gnavus* or *Dorea formicigenerans*. Finally, group 4 is formed by NAL proteins with low sequence identity from members of the family *Vibrionaceae*, *Shewanelaceae*, *Psychromonadaceae*, *Pseudoalteromonaceae* and some species of the genera *Bacteroides*, *Parabacteroides* and *Capnocytophaga*. (Figure 1.9)

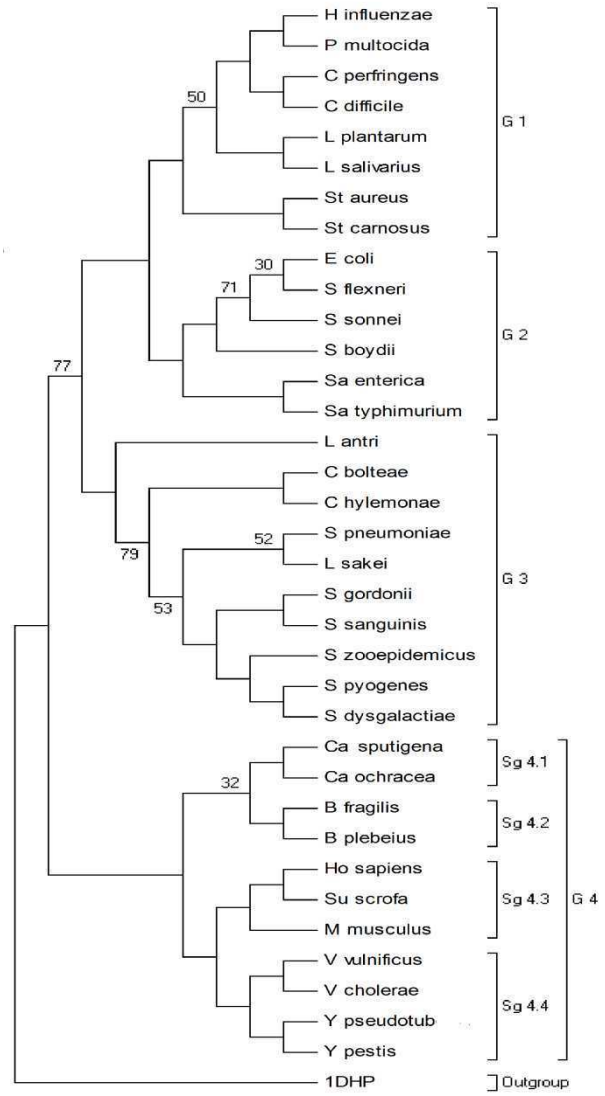


Figure 1.9: *Phylogenetic analysis of the bacterial NAL subfamily***.** H: *Haemophilus*, P: *Pasteurella*, C: *Clostridium*, L: *Lactobacillus*, St: *Staphylococcus*, E: *Escherichia*, S: *Shigella*, Sa: *Salmonella*, S: *Streptococcus*, S.pseudotub: *Streptococcus pseudotuberculosis*, Ca: *Capnocytophaga*, B: *Bacteroides*, Ho: *Homo,* Su: *Sus,* M: *Mus,* V: *Vibrio*, Y: *Yersinia*, G: group, Sg: subgroup (Sánchez-Carrón *et al*., 2011).

1.4.2 N-acetylneuraminte synthase

N-acetylneuraminte synthase (NAS; E.C. 2.5.1.56) catalyzes the irreversible condensation of phosphoenolpyruvate (PEP) with either N-acetylmannosamine (bacteria) or N-acetylmannosamine-6-phosphate (mammals) to give N-acetylneuraminic acid or N-acetylneuraminic acid-6-phosphate, respectively (Figura1.10) (Tanner, 2005). NAS has previously been purified or cloned from *E .coli* K1 (Vann *et al*., 1997; Hwang *et al*., 2002), *Streptococcus agalitiae* (Suryanti *et al*., 2003), *Neisseria meningitides* (Gunawan *et al*., 2005; Blacklow and Warren, 1962) and *Campylobacter jejuni* (Sundaram *et al*., 2004). Enzymes from eukaryotes have also been studied, including human (Lawrence *et al*., 2000), *Drosophila melanogaster* (Kim *et al*., 2000) and rat (Chen *et al*., 2002).

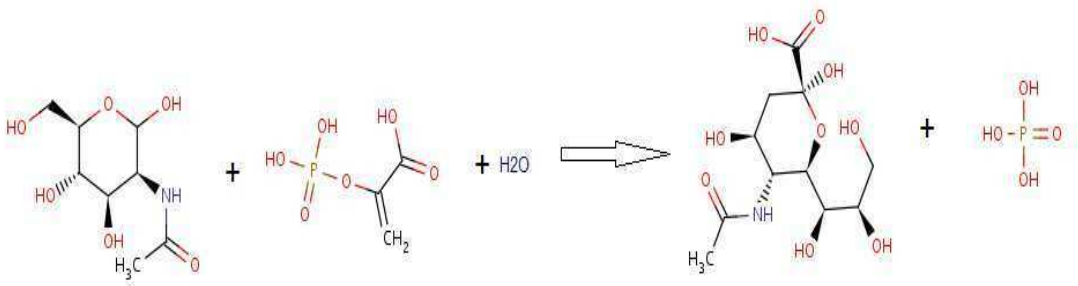


Figura 1.10: *Reaction catalyzed by N-acetylneuraminate synthase*

These enzymes do not accept mannose as an alternate substrate. All of the sialic acid synthases studied until now appear to be metalloenzymes that require a divalent cation for activity, and most show the highest activity in the presence of Mn^{+2} (Tanner, 2005).

1.4.2.1 Mechanism of action

Two potential mechanisms have been considered for sialic acid synthase, which catalyze the condensation of PEP with an aldehyde. In the first mechanism, the initial step involves an attack by the C-3 of PEP on the carbonyl carbon of ManNAc (Figure 1.11). This type of mechanism is well documented in two other systems that catalyze a very similar reaction and that have received a much attention, 2-keto-3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (DAHP synthase) and 2-keto-3-deoxy-D-*manno*-octulosonate-8-phosphate synthase (KDO-8P synthase) (Liang *et al*, 1998; Shumilin *et al*., 2004) (Figure 1.11). A second possible mechanism involves an initial attack of water on the phosphorus of PEP to give free phosphate and the enolate of pyruvate (Tanner, 2005) (Figure 1.11).

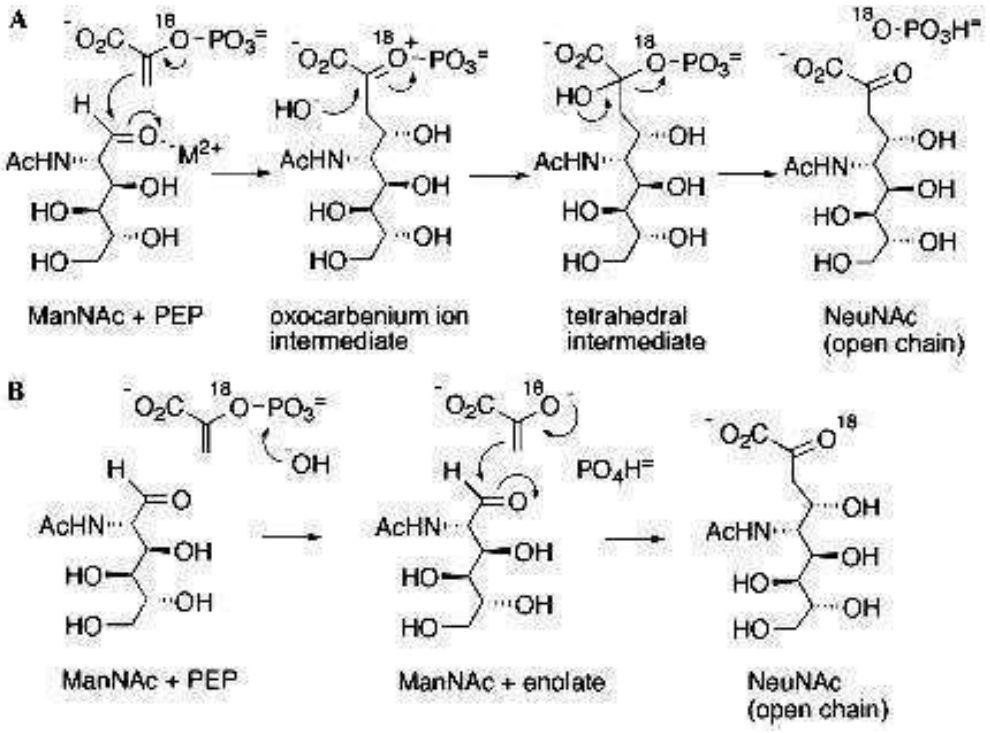


Figure 1.11: *Two potential mechanisms for the reaction catalyzed by sialic acid synthase.* (A) A C-O bond cleavage mechanism. (B) A P-O bond cleavage mechanism.

1.4.2.2 Structure

To date, only the X-ray structures of NeuAc synthase from *Neisseria meningitidis* (PDB:1xuu) has been solved (Gunawan *et al*., 2005). The enzyme was crystallized in the presence of Mn^{2+}, PEP, and the unreactive substrate analog *N*-acetylmannosaminitol (rManNAc or reduced ManNAc). Each monomer of the enzyme contains two domains joined by an extended linker region (Figure 1.12). The larger N-terminal domain has the classic TIM barrel fold of an eight-stranded β-barrel enclosed by eight helices. The smaller C-terminal domain is comprised of 65 residues in a "pretzel-shaped" fold that is remarkably similar to those found in fish type III antifreeze proteins (Jia *et al*., 1996). Two such monomers arrange themselves to form a domain-swapped homodimeric architecture in which the active sites the C-terminal portion of the TIM barrel from one monomer capped by the antifreeze domain of the opposite monomer (Figure 1.12). The antifreeze domain donates one key residue, Arg-314, directly to the active site, where it forms a hydrogen bond with the acetyl oxygen of rManNAc (Tanner, 2005).

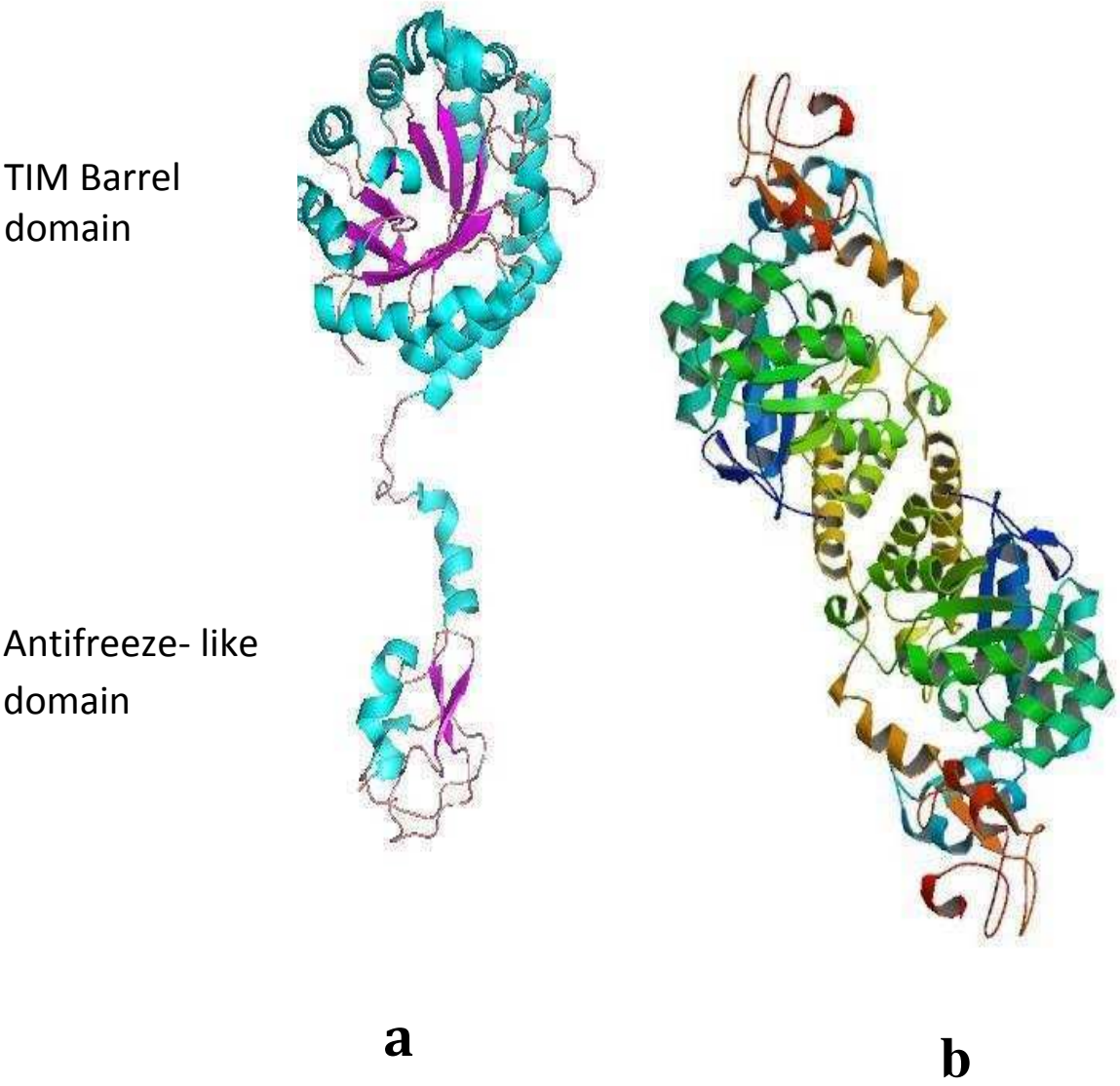


Figure 1.12: Neu5Ac synthase from *Neisseria meningitides.* Ribbon representation (a) monomer form (b) dimeric form (Pdb code: 1xuu; Gunawan *et al*., 2005).

1.5 IMMOBILIZATION OF ENZYMES

Enzymes are widely applied in many different industries and the number of applications continues to increase. Examples include food (baking, dairy products, starch conversion) and beverage (beer, wine, fruit and vegetable juices) processing, animal feed, textiles, pulp and paper, detergents, biosensors, cosmetics, health care and nutrition, waste water treatment, pharmaceutical and chemical manufacture and, more recently, biofuels production such as biodiesel. The main driver for the widespread application of enzymes is their small environmental footprint.

Enzymes are not widely used in industrial chemical processes because most are not stable in the working conditions. In addition, to being water soluble, their separation from the substrates and products is difficult, and therefore cannot be reused.

The development of new systems, involving the immobilization of enzymes has allowed many of these problems to be solved.

1.5.1 General aspects of enzyme immobilization.

The immobilization of enzymes is a process in which to the enzyme is localized in a defined region, resulting in insoluble forms, which retain their catalytic activity and can be recovered from the medium and reused.

Advantages of immobilized enzymes (Hartmeier., 1985):

- Reuse
- Continuous use
- Less labor intensive
- Saving in capital cost
- Minimum reaction time

- Less chance of produced contamination
- Greats stability
- Improved process control and high enzyme: substrate ratio.

Disadvantages of enzyme immobilization (Martinek and Mozhaer, 1997):

• Possibility of altering the conformation of the enzyme compared to its native state.

• Heterogeneity of the enzyme-support system in which be different fractions of proteins may be immobilized with a different number of bonds to the substrate.

• Frequent a loss of enzyme activity during mobilization.

• Cost. The biocatalyst is more expensive than the native enzyme.

1.5.2 Methods of immobilization.

There are basically, three traditional methods of enzyme immobilization:

- Binding to a support (carrier)
- Entrapment (encapsulation)
- Cross-linking

1.5.2.1 Binding to a support

Support binding can be physical, ionic, or covalent in nature. However, physical bonding is generally too weak to keep the enzyme fixed to the carrier in industrial conditions of high reactant and product concentrations and high ionic strength. The support can be a synthetic resin, a biopolymer or an inorganic polymer such as (mesoporous) silica or a zeolite.

1.5.2.2 Entrapment

Entrapment involves inclusion of an enzyme in a polymer network (gel lattice) such as an organic polymer or a silica sol-gel, or a membrane device such as a hollow fiber or a microcapsule. Entrapment requires the synthesis of the polymeric network in the presence of the enzyme.

1.5.2.3 Cross-linking

Cross-linking of enzymes aggregates or crystals, using a bifunctional reagent, to prepare carrier-free macroparticles. The use of a carrier inevitably leads to a 'dilution of activity', owing to the introduction of a large portion of non-catalytic ballast, ranging from 90% to >99%, which results in lower space-time yields and productivities. Moreover, immobilization of an enzyme on a carrier often leads to a substantial loss of activity, especially at high enzyme loadings.

Consequently, there is an increasing interest in carrier-free immobilized enzymes, such as cross-linked enzyme crystals (CLECs) and cross-linked enzyme aggregates (CLEAs) that offer the advantages of highly concentrated enzyme activity combined with high stability and low production costs because no additional (expensive) carrier is needed (Cao *et al*., 2003).

The use of cross-linked enzyme crystals (CLECs) as industrial biocatalysts was pioneered by Altus Biologics in the 1990s. CLECs proved to be significantly more stable to denaturation by heat, organic solvents and proteolysis than the corresponding soluble enzyme or lyophilized (freeze-dried) powder. CLECs are robust, highly active immobilized enzymes of controllable particle size, varying

from 1 to 100 μm. Their operational stability and ease of recycling, coupled with their high catalyst and volumetric productivities, renders them ideally suited for industrial biotransformations. However, CLECs have an inherent disadvantage: enzyme crystallization is a laborious procedure, requiring enzyme of high purity, which translates into prohibitively high costs.

The more recently developed cross-linked enzyme aggregates (CLEAs) , on the other hand, are produced by simple precipitation of the enzyme from an aqueous solution as physical aggregates of protein molecules, by the addition of salts or water miscible organic solvents or non-ionic polymers (Sheldon *et al*., 2004; Sheldon 2007). The physical aggregates are held together by non-covalent bonding without perturbation of their tertiary structure, that is, without denaturation. Subsequent cross-linking of these physical aggregates renders them permanently insoluble, while maintaining their pre-organized superstructure, and, hence their catalytic activity. This discovery led to the development of a new family of immobilized enzymes: cross-linked enzyme aggregates (CLEAs). Since precipitation from an aqueous medium, by addition of ammonium sulfate or polyethylene glycol, is often used to purify enzymes, the CLEA methodology essentially combines purification and immobilization into a single unit operation that does not require a highly pure enzyme. (Figure 1.13)

CLEAs are very attractive biocatalysts, owing to their straight forward, inexpensive and effective production method. They can readily be reused and exhibit improved stability and performance. The methodology is essentially applicable to any enzyme, including cofactor dependent oxidoreductases (Sheldon *et al*, 2007).

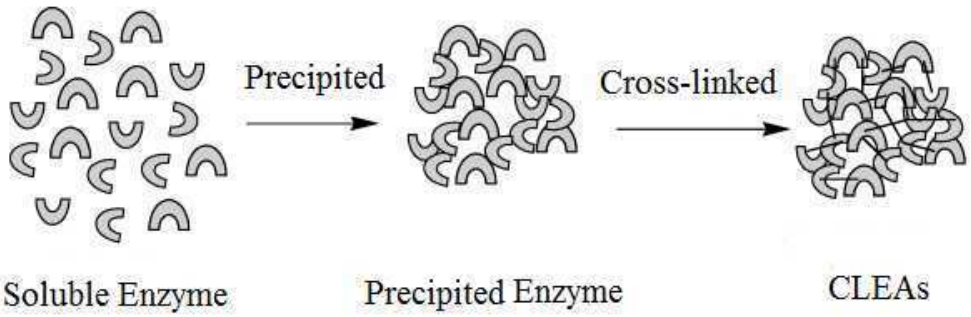


Figure 1.13: *Schematic representation for production of CLEAs.*

II. Objetives

The main objective of the present Doctoral Thesis was to explore new enzymes involved in the synthesis of sialic acid or Neu5Ac. In fact, the study was focused in two key metabolic enzyme related with the synthesis (N-acetyl neuraminate synthase) and the hydrolysis (N-acetyl neuraminate lyase) of sialic acid in order to find new biocatalyst from non-pathogenic microorganism to produce Nue5Ac and its derivatives. This main objective could be divided in the following partial objectives.

1) Molecular characterization and immobilization of a N-acetyl neuraminate lyase from *Lactobacillus plantarum* WFCS1 to produce Neu5Ac.
2) Molecular characterization of a N-acetyl neuraminate lyase from *Staphylococcus carnosus* to produce Neu5Ac.
3) Immobilization of N-acetyl neuraminate lyase from *Staphylococcus carnosus* to produce a Neu5Ac derivate, the 2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid (KDN)
4) Molecular characterization of N-acetyl neuraminate lyase from a non-studied phylogenetic group (group 3), in order to test them as possible biocatalyst for Neu5Ac synthesis.
5) Molecular characterization of new N-acetyl neuraminate synthase from a non-pathogenic microorganism.

III. Materials and Methods

3. MATERIAL AND METHODS

3.1 GENOMIC DNA

Genomic DNAs to obtain the different enzymes used in this thesis were from:

1. Lactobacillus plantarum WFCS1. It was used as the source of a putative N-acetylneuraminate lyase. The microorganism was first cultivated in MRS medium (Pronadisa, Condalab, Spain). DNA extraction was carried out using DNeasy tissue extraction kit from Qiagen (Valencia, USA).

2. Staphylococcus carnosus. It was used as the source of a putative N-acetylneuraminate lyase. The microorganism was first cultivated in Trypticasein soy broth-Agar medium at 30 ºC for three days. Then, one colony was picked in Trypticasein soy broth-Agar liquid medium and incubated at 30 ºC overnight. DNA extraction was carried out using DNeasy tissue extraction kit from Qiagen (Valencia, USA). *3. Lactobacillus sakei 23K*. Its genomic DNA was kindly provided by Prof. (Monique Zagorec, Unité Flore Lactique et Environnement Carné, INRA, Domaine de Vilvert, F-78350 Jouy-en-Josas, France), and was used as the source of N-acetylneuraminate lyase gene.

4. Lactobacillus antri. Its genomic DNA was directly provided by German culture collection (DSMZ) (#16041), and was used as the source of N-acetylneuraminate lyase gene.

5. Idiomarina lohiensis L2TR. Its genomic DNA was directly provided by German culture collection (DSMZ) (#15497), and was used as the source of N-acetylneuraminate synthase gene.

3.2 REACTIVES AND COMERCIAL STRAINS

Enzymes substrates (D-arabinose, L-gulose, D-lyxose, 2-deoxy-D-glucose D-mannose, *N*-acetylmannosamine and *N*-acetylneuraminic acid) were purchased from Carbosynth (Berkshire, UK), cofactors (NAD^+, NADH and ATP), divalent ions ($CaCl_2$, $CoCl_2$, $MnCl_2$, $MgSO_4$), monovalent ions (NaCl and KCl), EDTA, IPTG, antibiotics (kanamycin, ampicillin and chloramphenicol), molecular mass standard for gel filtration chromatography, bovin serum albumin (BSA) for protein quantification, glutaraldheyde and sodium borohydride were purchased from Sigma-Aldrich (Madrid, Spain).

Ammonium sulfate and inorganic buffers were obtained from Fluka. Electrophoresis and protein quantification (Bradford) reactives were from Biorad (Hercules, USA). HPLC disolvents were from Análisis Vínicos (Tomelloso, Spain). Purified water was obtained through a MilliQ system (Millipore, Bedford, USA). Culture media were acquired from Pronadisa (Condalab, Madrid, Spain).

Restriction enzymes, antarctic phosphatase (CIP), deoxyribonucleotides (DNTPs) and *Taq* polymerase were obtained from New England Biolabs (Berverly, MA, USA). *Pfu* Ultra II polymerase was purchased from Stratagen (Amsterdam, The Netherlands) and T_4 DNA ligase was from Roche (Manheim, Germany). The oligonucleotides specifically designed for each cloned gene were obtained from Isogen Life Sciences (Darmstad, Germany). Cloning strain DH5α was from Invitrogen (Frederick, USA). Expression vectors and *Escherichia coli* strains for protein expression used were obtained from Novagen (Darmstadt, Germany) (Table 3.1 and 3.2).

Table 3.1: E. coli *strains used in this work.*

Strain	Company	Utility
DH5α	Invitrogen	Cloning
Rosetta(DE3)pLys	Novagen	Expression
Rosetta(DE3) 2	Novagen	Expression

Table 3.2: *Cloning vectors used in this work.*

Plasmid	Company	Resistance	His-Tag	Utility
pET28a	Novagen	Kan^R	*N*-ter	Expression
pET523C/LIC	Novagen	Amp^R	*C*-ter	Expression

Molecular biology kits used in the purification of DNA (genomic, plasmid and PCR fragments) were purchased from Qiagen (Valencia USA). DNA recovery gel kit was obtained from Zymo (Irvine, USA). DNA quantification and purity were spectrophotometrically determined in a TrayCell (Hellma, Müllheim, Germany), through a spectrum between 220 and 300 nm.

3.3 BIOINFORMATIC TOOLS

Gene sequences were obtained from National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov/). Sequence alignment and identification of protein similarities were made by BLAST (Basic Local Alignment Search Tool) (Altschul *et al*., 1990). The sequences were aligned using ClustalW2 (Thompson *et al*., 1994) and ESPript (Gouet *et al*., 1999).

Protein sequences were 3D modelled with Geno3D (Combet *et al*., 2002) or i-TASSER (Roy *et al*., 2010).

Lysines and the Grand average hydropathicity index (GRAVY) were calculated using the ProtParam tool from Expasy Proteomic server (http://expasy.org/tools/protparam.html). Negative GRAVY values indicate hydrophilic proteins (Kyte and Doolittle, 1982).

Three different tree-building methods were used: Maximum Likelihood (ML), Bayesian analysis (BY), and Neighbor Joining (NJ) as implemented in PHYML and MrBayes 3.1.2, and MEGA 4, respectively (Felsenstein, 1989; Guindon & Gascuel, 2003; Huelsenbeck & Ronquist, 2001). The Bootstrap values for ML and NJ trees were obtained after 1000 generations. For the trees constructed using BY, the Markov chains were run for 1,000,000 generations. The burn-in values were set for 10,000 generations, and the trees were sampled every 100 generations. Splitstree and MEGA 4 tree viewer were used to visualize the trees and calculate confidence values (Tamura *et al*., 2007). The GC content of the sequences was calculated, and compared to the GC content of the whole genome, using the formulae previously described (Karlin, 2001). Functional divergence was tested using the DIVERGE software (Gu & Vander, 2002). The alignment used for the phylogenetic analysis was also used for this application.

3.4 MICROBIOLOGIC METHODS

3.4.1 Culture media, growing and conservation of strains

E. coli was grown at 37 ºC with agitation in solid or liquid Luria-Bertani medium (LB) (Pronadisa, Condalab, Spain). For high yield protein expression Terrific Broth (TB) (Pronadisa, Condalab, Spain) was used, supplemented with the necessary antibiotics, kanamycin (50 µg/mL) and chloramphenicol (34

μg/mL). For the induction of T7 RNA polymerase promoter, isopropyl-β-D-thiogalactoside (IPTG) was added to the cultures, in a final concentration of 1-1.5 mM. The composition of media was as follow:

Luria-Bertani (LB):

Tryptone	10 g/L
Yeast extract	5 g/
NaCl	10 g/L

Terrific Broth (TB):

Tryptone	12 g/L
Yeast extract	24 g/L
Potassium monophosphate	2.3 g/L
Potassium diphosphate	12.5 g/L
Glycerol (v/v)	4 ml/L

The dehydrated media were dissolved in distilled water and autoclaved at 121 ºC during 20 minutes, and then conserved at 4 ºC, until use. Once the culture was inoculated, its growth was followed spectrophotometrically (UV-2401PC, Shizamdzu Corporation, Japan), by measuring optical density at 600 nm (OD_{600}). When *E. coli* cells were stored for long periods, glycerol stocks (16 %) where storage at -80 ºC (UltraLow MDFU3086, Sanyo Electric, Japan)

3.4.2 Transformation

E. coli competent cells (DH5α and Rosetta) were transformed by the electroporation method (Dower *et al*., 1988), with an electroporator BioRad

(Hercules, USA). After transformation cells were recovered at 37 ºC in SOC medium (0.5 % yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) for 1 hour, before platting in the corresponding Petri dishes, containing the appropriate antibiotic.

3.5 PCR AMPLIFICATION AND CLONING OF DNA FRAGMENTS

In general terms, all the methods used for cloning are described in the book of Sambrock *et al*. (1989). The amplification of DNA fragments was made by PCR reaction using a thermocycler TGradient Biometra (MJ Research, USA). For each PCR reaction, specific oligonucleotides were used (100 µM), which were complementary to 5' and 3' ends of the target gene. Then, this PCR product was then cloned, using restriction enzymes, in a pET vector (T7 RNA polimerase promoter), which is inducible by IPTG. PCR conditions were as follow unless otherwise is indicated:

Table 3.3: *PCR standard conditions*

Step	Temperature (ºC)	Time
1	95	1 minute
2	95	30 seconds
3	54	45 seconds
4	72	1 minute/Kb
5	Repeat steps 2-4 (x29)	
6	72	10 minutes

3.5.1 Cloning of recombinant NAL of *Lactobacillus plantarum*

N-acetylneuraminate lyase gene, denoted as *nanA* (NCBI) was amplified by PCR (forward primer 5´-CAGGGACCCGGTA TGAGTAAAAAACTATTGTATGCAGCCCAAATG-3´ and reverse primer 5´-G GCACCAGAGCGTTGTTTTTGAAGTATTTTTCGTAAATCGCCG-3´), treated with LIC-qualified T4 DNA polymerase, inserted in pET52 3C/LIC vector and transformed into *E. coli* BL21(DE3)pLys competent cells (Novagen)

3.5.2 Cloning of recombinant NAL of *Staphylococcus carnosus*

N-acetylneuraminate lyase gene, denoted as *nanA* (NCBI) was amplified by PCR using *Pfu* Ultra II, and the following oligonucleotides containing restriction enzyme sites (underlined) for *NheI* in the 5' and *XhoI* in the 3': 5'-TATGGCTAGCATGGAAGAAAATTTGAAAGGAT-3' (forward primer) and 5'GCGCTCGAGTTACAAATTGTATTTATTTACTAAC-3' (reverse primer). The PCR product was digested with the same restriction enzymes, purified by QIAquick PCR purification kit (Qiagen) and inserted into the expression vector pET28a downstream of the T7 RNA polymerase promoter, which had been previously digested with the same restriction enzymes. This produced the recombinant plasmid named pET28-*ScNAL*, which encodes for an additional 36 amino acid *N*-terminal sequence containing a six histidine tag. Plasmids were transferred to *E. coli* DH5α. Kanamycin resistant clones were confirmed by colony PCR and analytic digestion. The positive clones were transformed in *E. coli* Rosetta (DE3)pLys, and the correct sequence was checked by DNA sequencing.

3.6 PROTEIN PURIFICATION

E. coli cells harboring the recombinant plasmid were grown for 4 hours at 37º C in 400 mL of LB Kan-Chlor before being transferred to a 5-L fermentor (Sartorius), containing 4 L of Terrific Broth, supplemented with the latter antibiotics. This culture was allowed to grow for 3h at 37 ºC, and then, induced by adding 1 mM isopropyl-β-D-thiogalactoside (IPTG), except in the case of LsNAL, which was 1.5 mM, for 12 hours at 30 ºC with constant stirring and oxygenation. The culture was diafiltered through a 500-kDa membrane (GE Lifesciences, Uppsala, Sweden), and cleaned with 50 mM of the appropriate buffer. Cells were disrupted using a homogenizer (MiniZetaII, Netzsch) and the cells debris were harvested by centrifugation. The recovered was treated with 3 U/mL DNase I (Sigma) to remove nucleic acids, and then centrifuged for 20 min at 6,000*g*.

The purification was performed in two steps, starting with tangential ultrafiltration with a 50-kDa cutoff membrane (for ScNAL) or 100 kDa cutoff membrane (for LpNAL, LsNAL, LaNAL and IiNAS) on step a QuixStand system (GE Lifesciences). After centrifugation at 40,000*g,* the resulting supernatant was purified by Ni^{2+}-chelating affinity chromatography (ÄKTA Prime Plus, GE Lifesciences) onto a HiPrep IMAC 16/10 FF 20 mL column (GE Lifesciences). The bound enzyme was eluted with a linear imidazol gradient up to 250 mM in the appropriate buffer at a flow rate of 10 mL/min. The fractions containing the desired activity were pooled, desalted, concentrated and stored at -20 ºC.

3.7 PROTEIN METODOLOGY

3.7.1 Protein quantification

Protein concentration determination was carried out using Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as standard for the calibration curve.

3.7.2 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

For the electrophoretic resolution of proteins, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) method was performed in the presence of the anionic detergent sodium dodecyl sulfate (SDS), as described by Laemmli *et al* (Laemmli, 1970). Gel of 1.5 mm thick was prepared as follows: 12% (w/v) acrylamide-bis-acrylamide in 375 mM Tris-HCl pH 8.8, 0.1 % (w/v) SDS, 0.05 % (w/v) ammonium persulfate and 0.07 % (v/v) TEMED, being the two last components necessary to start the polymerization reaction. A comb was introduced in the 4% stacking gel [4 % (w/v) acrylamide-bisacrylamide in 125 mM Tris-HCl pH 6.8, 0.1 % (w/v) SDS, 0.05 % ammonium persulfate and 0.07 % (v/v) TEMED] to obtain the wells to load the samples.

Each sample (15 µL) was mixed with 5 µL of 4X loading buffer, whose composition was: 15 % (v/v) glycerol, 3 % (w/v) SDS, 0.015 % (w/v) bromophenol blue and 7.5 % (v/v) β-mercaptoethanol in 100 mM Tris-HCl buffer pH 6.8. Then, the samples were heated at 100 ºC for 4 minutes before loading them in the staking gel.

Electrophoresis was carried out in a Mini Protean III system (Biorad, Hercules, USA) at 200 V for 45 minutes using 25 mM Tris-HCl, 200 mM glycine, 0.1 % SDS (w/v) pH 8.3 as a running buffer. Proteins were stained with Brilliant Blue Coomasie (Sigma, Madrid, Spain) for 30 minutes, and then, the gel was

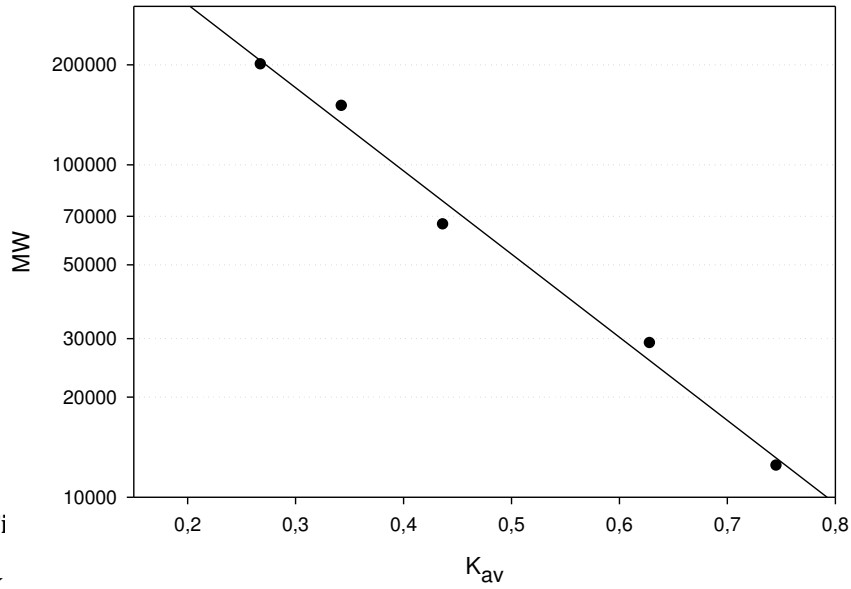
destained using a solution containing 30 % methanol and 10 % acetic acid. For determining the molecular mass of the protein under denaturing conditions, molecular weight standard P7708S (NEB, Beverly MA, USA) was used which included the following proteins: MBP-β-galactosidase, MW 175 kDa; MBP-paramyosin, MW 80 kDa; MBP-CBD, MW 58 kDa; CBD-*Mxe* Intein-2CBD, MW 46 kDa; CBD-*Mxe* Intein, MW 30 kDa; CBD-BmFKBP13, MW 25 kDa, lysozyme 17 kDa; aprotinin, MW 7 kDa.

3.7.3 Molecular mass determination

Monomeric molecular mass determination of the proteins were carried out under denaturing conditions, as described above, and by liquid chromatography-mass spectrometry with electrospray ionization, using an HP-1100 (Agilent Technologies), according to previously published methods (Pearcy & Lee, 2001; Williams *et al*., 2001). This procedure is based on the previous separation of the sample in a HPLC HP-1100 (Agilent technologies, USA), which is coupled to a mass spectrometer ESI Trap (*Trap XCT Plus*, Agilent technologies, USA). Analysis by LC/MSD Ion Trap software (version 3.2) was used to calculate with an accuracy of about 1 Da. This analysis was performed in the Servicio de Proteómica of Centro de Ayuda a la Investigación y Desarrollo (CAID) of the University of Murcia.

Gel filtration (Superdex 200 10/300 GL, GE Lifesciences, Uppsala, Sweden) was used to confirm the homogeneity, and the molecular weight of the native purified enzyme. The column was equilibrated with 50 mM Tris-HCl buffer pH 7.5, containing 0.15 M NaCl, and previously calibrated using standard proteins of know molecular weight (Figure 3.2). The chromatography was performed in

an ÄKTA purifier FPLC (GE Lifesciences, Uppsala, Sweden) at a flow rate of 0.5 mL/min.



Fi *sing gel filtration*. $K_{av}=(Ve-Vo)/(Vt-Vo)$, where K_{av} is the phase distribution coefficient, Ve is the elution volume, Vo is the death volume (calculated with blue dextran) and Vt is the total volume of the column. Proteins used were: Blue Dextran, 2000 kDa; β-amilase, 200 kDa; Alcohol dehydrogenase, 150 kDa; Albumin bovine serum, 66 kDa; Carbonic anhydrase, 29 kDa and Cytochrome C 12.4 kDa.

3.7.4 Protein unfolding: determination of melting temperature (T_m)

Melting curves to determine protein unfolding were obtained with the fluorescent dye SYPRO Orange (Molecular Probes, Paisley, UK). SYPRO Orange is a hydrophobic, environmentally sensitive fluorophore, that is quenched in aqueous solutions, but which binds to exposed hydrophobic surfaces of an unfolding protein, leading to a sharp increase in fluorescence emission as a function of temperature. 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 calorimetry (DSC) (Ericsson *et al*., 2006). 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 real time-PCR machine (model 7500, Applied Biosystems). The time/temperature control of the PCR machine was adapted from Malawski *et al* (2006), and consisted of 70 steps of 1 min each, raising the temperature by 1 °C steps, from 20 to 90 °C. Independent experiments were conducted with a minimum of three replicates per condition. This technique was used not only to determine the thermal protein stability, but also its pH stability.

3.8 SPECTROPHOTOMETRIC ASSAYS

3.8.1 Enzyme assay for N-acetylneuraminate lyase

The conversion of Neu5Ac into ManNAc and pyruvate by NALs (ScNAL, LsNAL and LaNAL) was determined using lactate dehydrogenase as coupled enzyme (E.C: 1.1.1.27). This method measured the decrease in absorbance at 340 nm corresponding to the oxidation of the NADH (ε_{340} = 6,200 M^{-1} cm^{-1}), produced by lactate dehydrogenase (LDH), during which pyruvate appeared as a consequence of the hydrolysis of Neu5Ac into such compound and ManNAc by NALs. The standard reaction medium (1 mL) for the above assay at 37 ºC, which was carried out in a Shizmadzu UV-2401PC spectrophotometer, contained 150 µM NADH, 0.5 U LDH, 10 mM Neu5Ac and purified NALs (4 µg ScNAL, 17 µg LsNAL and 1 µg LaNAL) in 20 mM sodium phosphate buffer pH 7. A control

assay without Neu5Ac was also carried out to determine the presence of any other NADH-consuming enzymes. One unit of activity was defined as the amount of enzyme required to consuming 1 μmol of NADH in 1 per minute under the standard assay conditions.

3.9 HPLC ASSAYS

3.9.1 Enzyme assay for N-acetylneuraminate lyase

The conversion of ManNAc and pyruvate into Neu5Ac was determined by HPLC-ELSD II (Shimadzu) using an Amino UK column (Imtakt Co. Japan) and a mobile phase (58 % acetonitrile: 42 % 50 mM ammonium acetate) running at 0.4 mL/min at 60 ºC. In these conditions, the retention time (R_T) for Neu5Ac and ManNAc were 10.3 and 4.2 min, respectively. The synthetic reaction using ScNAL was followed using the above HPLC conditions and contained, in the standard reaction medium, 300 mM ManNAc, 50 mM pyruvate and 20 μg purified ScNAL in 20 mM phosphate buffer pH 7.0. The standard reaction medium for the synthetic reaction using LaNAL was followed the above HPLC conditions and contained of 500 mM ManNAc, 30 mM pyruvate and 50 μg purified LaNAL in 20 mM phosphate buffer pH 7.0. In case of LsNAL the conditions were 500 mM ManNAc, 10 mM pyruvate and 2 μg purified LsNAL in 20 mM phosphate buffer pH 7.0. One enzymatic unit was defined as the the amount of enzyme required to synthesize 1 μmol of Neu5Ac per minute under above conditions. After 15 minutes of incubation at 37 ºC, the reaction was stopped with 1 volume of acetonitrile, and then incubated on ice for 1 hour for protein precipitation. After precipitation, the mixture was centrifuged at 13000*g* for 30 min and filtered through a 0.2 μm filter. One unit was defined as

the amount of enzyme required to convert 1 µmol of ManNAc and pyruvate to Neu5Ac per minute, under above conditions.

3.10 KINETIC CHARACTERIZATION

3.10.1 Temperature effect

The thermostability of the enzyme was examined using the standard assay for each enzyme after incubation of the enzyme from 40 ºC to 80 ºC for different periods of time (from 5 minutes to 24 hours, depending on the enzyme stability). When a stabilizer was tested, it was added to the enzyme during the incubation period at the appropriate final concentration.

3.10.2 pH effect

The optimum pH of the purified enzyme was studied from pH 4.0 to 11.0 in its corresponding reaction medium and using the following 20 mM buffers: sodium acetate (pH 5.0 to 5.5), sodium phosphate (pH 6.0 to 8.0), Tris-HCl (pH 8.5 to 9.0) and glycine (pH 9.5 to 11.0). In the pH stability study, the residual enzyme activity was measured after 0.5 to 24 hours of incubation at 37 ºC in its corresponding standard reaction medium and in the same buffers as described above.

3.11 PRODUCTION OF CROSS-LINKED ENZYME AGGREGATES (CLEAS)

Enzymes are widely applied in many different industries and the number of applications continues to increase. Examples include food (baking, dairy products, starch conversion) and beverage (beer, wine, fruit and vegetable juices) processing, animal feed, textiles, pulp and paper, detergents, biosensors, cosmetics,

health care and nutrition, waste water treatment, pharmaceutical and chemical manufacture and, more recently, biofuels such as biodiesel. The main driver for the widespread application of enzymes is their small environmental footprint.

The use of enzymes is not widely used in industrial chemical processes because most enzymes are not stable in the working conditions. In addition, to being water soluble, its separation from the substrates and products is difficult, and therefore cannot be reused.

The development of new systems immobilization of enzymes has allowed resolving these problems.

The immobilization of enzymes is a process in which it is localized to the enzyme in a defined region of space, resulting in insoluble forms which retain their catalytic activity and can be recovered from the medium and reused.

Advantages of immobilized enzymes:

- reuse
- continuous use
- less labor intensive
- saving in capital cost
- minimum reaction time
- less chance of contamination in products
- more stability
- improved process control and high enzyme : substrate ratio.

Basically, three traditional methods of enzyme immobilization can be distinguished, Binding to a support (carrier), Entrapment (encapsulation) and

Cross-linking. Support binding can be physical, ionic, or covalent in nature. However, physical bonding is generally too weak to keep the enzyme fixed to the carrier under industrial conditions of high reactant and product concentrations and high ionic strength. The support can be a synthetic resin, a biopolymer or an inorganic polymer such as (mesoporous) silica or a zeolite. Entrapment involves inclusion of an enzyme in a polymer network (gel lattice) such as an organic polymer or a silica sol-gel, or a membrane device such as a hollow fiber or a microcapsule. Entrapment requires the synthesis of the polymeric network in the presence of the enzyme. The last method to immobilized enzyme, not need support.

The use of a carrier inevitably leads to 'dilution of activity', owing to the introduction of a large portion of non-catalytic ballast, ranging from 90% to >99%, which results in lower space-time yields and productivities. Moreover, immobilization of an enzyme on a carrier often leads to a substantial loss of activity, especially at high enzyme loadings.

Consequently, there is an increasing interest in carrier-free immobilized enzymes, such as cross-linked enzyme crystals (CLECs) and cross-linked enzyme aggregates (CLEAs) that offer the advantages of highly concentrated enzyme activity combined with high stability and low production costs owing to the exclusion of an additional (expensive) carrier (Cao *et al*., 2003).

CLEAs are aggregates of enzymes precipitated by salts, organic solvents or polymers nonionic. These polymers can be crosslinked chemically below by the addition of "cross-linkers", such as glutaraldheyde, this dialdehyde is safe, cheap and easy to handle. This compound forms covalent bonds between the amino groups (-NH2) of lysine residues. (Figure 3.1). This cross linking stabilizes the

structure of these aggregates making it easier physical handling and returning more resistant enzyme generally to temperature changes, pH, organic solvents or proteolysis.

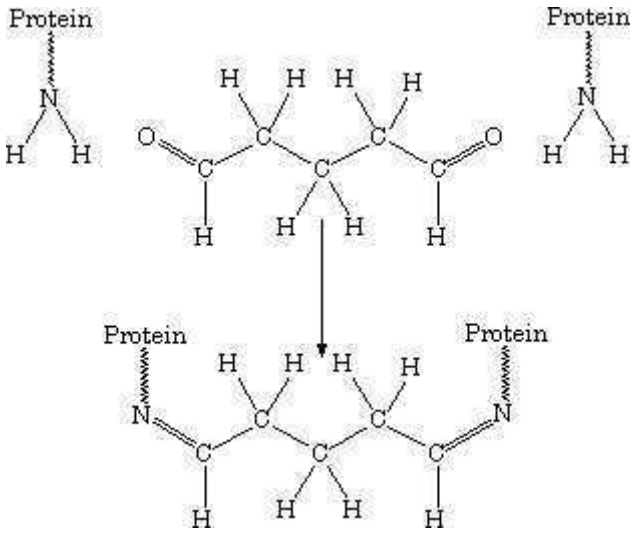


Figure 3.3: *Crooslinking enzyme aggregates by means of gulutaraldehido addiction*. This union is between the amino groups of the lysines of the enzyme and crosslinker by Schiff base.

3.11.1 CLEAs production

Aggregation tests with ammonium sulfate and the following steps were carried out at 4 ºC by adding to 1 mL of a 10 mg/mL pure NALs in 20 mM sodium phosphate buffer pH 7.5, the required volume of saturated ammonium sulfate solution to bring the mixture to the desired degree of saturation with the salt. Pilot aggregation tests with acetonitrile or with *tert*-butanol (final concentrations up to 90% v/v) were carried out in the same way, except that cold solvent was added. After one hour, the appropriate amount of a freshly prepared 25% (v/v) aqueous solution of glutaraldehyde was stirred into the suspensions to attain the desired concentration of the dialdehyde (concentrations tested, 0.2, 0.5, 1 or 2% glutaraldehyde). At various times after the addition of the glutaraldehyde, 100-µL

aliquots were suspended in 900 µL of 20 mM potassium phosphate pH 7.5 and were centrifuged (16000*g*; 2 min), after which the enzyme activity in the supernatant and in precipitates was determined. Test assays yielded active aggregates when using ammonium sulfate at 90% saturation and a 4-hour cross-linking period with 1% glutaraldehyde (AS-CLEAs),.

At the end of the cross-linking period the entire suspension was centrifuged (16000*g* x 2 min), washing the precipitated CLEAs three times by repeated cycles of suspension in 20 mM sodium phosphate pH 7.5 and centrifugation.

Sodium borohydride CLEAs (SB-CLEAs) were prepared as above, except that at the end of cross-linking step the whole reaction volume was doubled with 0.1 M sodium bicarbonate buffer pH 10, and during 30 min, 1 mg/mL of $NaBH_4$ was added at intervals of 15 min (Wilson *et al*., 2004). The resulting CLEAs were washed and stored as AS-CLEAs.

Finally, BSA-CLEAs were formulated by slowly adding ammonium sulphate at 90% saturation to an LpNAL solution (10 mg/mL) containing 2 mg/mL of bovine serum albumin under stirring conditions and a 4-hour cross-linking period with 1% glutaraldehyde. Then, the whole reaction volume was treated with sodium borohydride as described for SB-CLEAs. All the preparations of the different CLEAs described above were stable for several weeks when stored at 4 ºC.

3.11.2 Enzyme assays with CLEAs

For the standard assay of the synthetic activity of N-acetyl-D-neuraminc acid aldolase, i.e. obtaining Neu5Ac from pyruvate and ManNAc, the enzyme (generally, 0.1 mg/mL of CLEAs) was incubated at 37ºC with the above substrates at the indicated concentration of 100 mM ManNAc and 30 mM Pyr in 20 mM sodium

phosphate pH 7.5. The reaction was stopped by centrifuging out the CLEAs (16000*g* x 2min). The sample from the supernatant were subjected to normal-phase HPLC (LC-20A, Shimadzu) using an AMINO-UK column (Imtakt Corporation, Kyoto, Japan), as described in section 3.9.1. One unit of activity was defined as the amount of enzyme required to synthesize 1 μmol of Neu5Ac per minute under the above conditions. Enzymatic activity was obtained from three repeated experiments.

3.11.3 Enzyme stability assay

The pH-stability was monitored by incubating the enzyme (either in soluble or in CLEA form) in a solution of 20 mM glycine buffer pH 9.0 at 50 ºC, taking samples after the periods of time specified to determine enzyme activity in the standard assay at 37 ºC, using ManNAc and pyruvate as substrates. In a similar way, the thermal stability was recorded by incubating the enzyme (in a free and aggregates form) in a solution of 20 mM glycine buffer pH 9.0 at the indicated temperatures (40-80 ºC). After cooling, enzyme activity was determined in standard assay at 37 ºC.

A pH-stability assay was carried out by incubating the enzyme in presence of 5 mM of sodium pyruvate and absence of this at various pHs (7.0-9.0) at 37 ºC. Aliquots of 100 μL were taken at different times and spectrophotometrically measured using *N*-acetyl-D-mannosamine dehydrogenase as coupled enzyme (E.C: 1.1.1.233). This method measured the increase in absorbance at 340 nm corresponding to the reduction of NAD^+ when the ManNAc produced by LaNAL is oxidized by the dehydrogenase to its corresponding lactona. We use this method to avoid possible interference from sodium pyruvate which added to the reaction and generated by the enzyme in presence of Neu5Ac.

3.11.4 Scanning electron microscopy of CLEAs

To study the external structure of the CLEAs produced in different treatments, use this microoscopia due to its high resolution (24 times the MO). It was not necessary due to treatment prior fixation due to glutaraldehyde (1%) treatment of crosslinking. The preparation of material for SEM requires a laborious method. The samples have completely dehydrated, drain and metalized to be studied in a high vacuum (10-5 Torr), where you can control the electron beam. Complete dehydration of the CLEAs was carried out using a gradient of increasing concentrations of acetone (v / v) (30%, 50%, 70%, 90% and 100%), the sample remaining on each concentration 10 min. The next step is the drying of the samples, this step is extremely important, to the preservation of the shape and structure of the particles. Technical "critical point" was used (Anderson, 1951), wherein the latter compound dehydration (liquid agent) is displaced by CO_2 on liquid (liquid transition), which at a temperature of 31 ° C and a pressure of 73.8 bar, it sublimates quickly without producing surface tension thereby maintaining the three dimensional structure of the particles. Liquid CO_2 is soluble in acetone, so that the latter will be displaced. For critical point drying was used UNION Balzer CPD 020 (Balzer Union Ltd, USA). Both dehydratations as the critical point technique were performed in the Microscopy Service (SACE, University of Murcia). After drying, the particles are placed in aluminum pedestals ("*stubs*") under a binocular microscope. The pedestals are provided with a conductive adhesive tape duplexing, place with the help of a micromanipulator particles in powder form. These pedestals are placed, then in a metalize. These pedestals are placed in a metalize CLEAs to cover with a thin gold layer, this technique is known as

("sputtering"). The metalizing (BIO-RAD Polarondivision Sputter Coat (USA) coated samples with a gold layer of 200 Å. Samples were processed and observed in the SEM Jeol (Japan), model JSM 6100 of SACE, an acceleration voltage of 15 kV electrons. SEM images were captured digitally.

3.11.5 CLEA dispersion and sizing of the resulting particles

Two methods were used for the disruption of the aggregates. One approach, which is widely used, consisted of vortexing at medium speed with a Heidolph (Germany) Reax Control vortex. In the other (novel) approach, the CLEAs were subjected to reciprocating mixing using a FastPrep-24® sample preparation system (M.P. Biomedicals, CA, USA) at a setting of 6.0 m/s (Montoro-García *et al*., 2010). The degree of dispersion attained by both procedures was monitored by bright-field optical microscopy at a final enlargement of 40X, taking digital images that were analyzed planimetrically by image processing (MIP 4.5 image analysis software, Digital Image System, Barcelona, Spain).

Chapter IV

Molecular characterization of N-acetyl neuraminate lyase from *Lactobacillus plantarum* WCFS 1 and its immobilization as FastPrep CLEAs to obtain Neu5Ac

4.1 INTRODUCTION

Sialic 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 (sialic acid, Neu5Ac) to pyruvate and *N*-acetyl-D-mannosamine, with an equilibrium that favours Neu5Ac cleavage. The enzyme plays an important role in the regulation of sialic acid metabolism in bacteria (Schauer, 1982; Traving *et al.*, 1997; Vimr and Troy, 1985b). Neu5Ac lyase has been found in pathogenic as well as non-pathogenic bacteria (Aisake *et al.*, 1991), and in mammalian tissues. Neu5Ac lyase also catalyzes the reverse aldol condensation reaction and has been used in this way to synthesize sialic acid and some of its derivatives from pyruvate and *N*-acetyl-D-mannosamine (Huang *et al*, 2007; Wang & Lee, 2005; Yu & Chen, 2006; Yu *et al*., 2004). Interest in this aspect of the enzyme's activity has increased with growing appreciation of the role of sialic acid in controlling biomolecular interactions, particularly at the cell surface.

Lactobacillus plantarum is a Gram-positive lactic acid bacterium, commensal of the human gastro-intestinal tract, which has a long history of safe use in many fermented sausages and fermented lactic products. *Lactobacillus* has also been used as a probiotic supplement or as an approved food additive that enjoys Generally Recognized as Safe (GRAS) status (Title 21 of the Code of Federal Regulations (21 CFR) and the FDA Office of Premarket Approval lists microorganisms). These characteristics make the *Lactobacillus plantarum* genome ideal for obtaining new recombinant enzymes, such as *N*-acetylneuraminate lyase, which can be safely used in industrial production processes. Although biocatalysts are useful tools for a green and sustainable industry (Sheldon, 2010), their synthetic application is often limited by production and operating costs. Numerous

efforts have been devoted to producing robust immobilized catalysts by binding enzyme to a solid carrier, by encapsulation in a organic or inorganic polymeric gel, or by cross-linking of the protein molecules in the form of cross-linked enzyme crystals (CLECs) or cross-linked enzyme aggregates (CLEAs) (Roessl *et al*., 2010). The latter method not only avoids the inherent dilution of the enzyme in the presence of an inert carrier, but also the need for a highly purified enzyme to start the laborious screening to identify conditions of crystallization that produce catalytic crystals (CLECs). A simple way of preparing CLEAs consists of protein precipitation into an aqueous solution by adding a salt, a water-miscible organic solvent or a polymer (Gupta *et al*., 2009). In a subsequent step, physical aggregates of the enzyme are cross-linked with a bifunctional agent, the agent of choice being glutaraldehyde, since it is inexpensive and readily available in commercial quantities (Sheldon, 2007a). Such cross-linking produces CLEAs, in which the catalytic activity of an individual enzyme is preserved (Sangeetha & Abraham, 2008). Since precipitation is frequently used to purify enzymes, this technology offers the possibility of using semipurified enzymes as starting material, thus reducing costs. In addition, multiple enzyme activities can be simultaneously captured in such aggregates (combi-CLEAs), for cascade or non-cascade conversion (Dalal *et al.*, 2007).

To exploit to its full the simplicity and robustness of CLEAs technology, several key issues need to be resolved, including how to control the particle size of the enzyme aggregates without causing significant diffusion constraints, how to avoid the dramatic modification of some essential Ɛ-amino groups by glutaraldehyde (which results in CLEAs with a significant loss of biological activity), and how to produce aggregates stable at basic pHs. Advance in the above critical questions have been

made since the first paper describing CLEAs (Cao *et al.*, 2000). These include the use of sodium borohydride in sodium bicarbonate buffer at pH 10 to reduce the Schiff base formed after cross-linking with glutaraldehyde (Wilson *et al.*, 2004), the use of alternative crosslinking agents, such as dextran-polyaldehyde (Mateo *et al.*, 2004) or bovine serum albumin (BSA) as a protein feeder (Shah *et al*., 2006) to prevent glutaraldehyde inactivation, and finally the use of longer vortexing periods (about one hour) to recover the enzyme activity lost during the centrifugation and cleaning steps needed to prepare CLEAs, which results in large aggregates due to the low compression resistance of CLEAs (Wang *et al.*, 2010a). These last mass transfer limitations may increase when co-aggregation with BSA is used, since the internal structure of the aggregates is disturbed, resulting in narrower channels that limit the diffusion of the substrate thought the CLEAs (Cabana *et al.*, 2007).

In this context, special attention should be paid to the two new methods developed to reduce the diffusional limitations of CLEAs. When the substrates of an enzyme are macromolecules, porous cross-linked enzyme aggregates (p-CLEAs) are prepared by adding starch as a pore-making agent, which is latter removed by α-amylase (Wang *et al.* 201b). In the case of small substrates, our group has described the use of a commercial cell distuptor (FastPrep®, www.mpbio.com), based on a precession movement to recover all the enzyme activity of an acetyl xylan esterase in a few seconds (Montoro-García *et al.*, 2010). This equipment produces a high-speed movement in all directions (vertical and angular motion) which causes cell disruption as a result to the collision between cells and beads in the reaction tube. The effectiveness of the cell disrupting process depends on the rate of the collision and the energy of the impact, which are functions of the speed settings (range, 4.0 to 6.5 m/s) and the specific gravity of the bead material used.

The rate of collision is proportional to the speed, while the energy of impact is proportional to the square of the speed (Müller *et al.*, 1998). In the case of CLEAs, their solid aggregates act as a dispersing material pulverizing each other in a short period of time (30 s) without the need for glass beads.

In order to expand the general use of such FastPrep-CLEAs (Fp-CLEAs) in biocatalysis, this methodology was applied to recover the activity of N-acetyl-D-neuraminic acid aldolase (E.C. 4.1.3.3., NAL), a model enzyme that shows all the main characteristic that need to be solved to produce CLEAs. Its activity decreased to 51% after protein crosslinking (0.5% glutaraldehyde) for 5 min, when cross-linked inclusion bodies (CLIBs) were formed (Nahálda *et al.*, 2008). This severe drop in activity could be due to the fact that the catalytic amino acid (lysine) in this aldolase is modified, as it has been described for nitrilase CLEAs, where its modification by glutaraldehyde lowered the activity to 49% (Mateo *et al.,* 2004). In addition, free NAL is used industrially at basic pHs (where standard CLEAs are not stable) for the condensation of pyruvate and N-acetyl-D-mannosamine (ManNAc) into N-acetyl-D-neuraminic acid (Neu5Ac), an advanced intermediate for obtaining GlaxoSmithKlime´s antiviral Relenza® (Liese *et al.*, 2006).

4.2. RESULTS

4.2.1 Optimization of CLEAs production

Pure *Lactobacullus plantarum* N-acetyl-D-neuraminic acid aldolase (LpNAL) was used for preparation of the aggregates as described in Materials and Methods, since this enzyme has great potential as a biocatalyst due to its high stability at alkaline pHs, its high over-expression in *E.coli* and its simple purification procedure (Sánchez-Carrón *et al.*, 2010). In a preliminary screen to select a

suitable agent for protein aggregation, three different substances were tested up to 90% concentration, either to change the hydration state of the enzyme molecules (ammonium sulfate) or to alter the electrostatic constant of the solution (acetone and acetonitrile). After one hour, the appropriate amount of glutaraldehyde (0.2-2%) was added, and the sample was diluted 10-fold in phosphate buffer and centrifuged to prove that no substantial activity remained in the supernatant after 4 hours crosslinking with any of the treatments. Only ammonium sulfate preserved the 40% of enzyme activity in the resulting CLEAs (AS-CLEAs) after 60-min vortexing (Table 4.1), whereas organic solvents eliminated the activity, pointing to a solvent-triggered denaturing effect. Similar findings have been reported for other CLEAs (Hara *et al*., 2008; Gupta *et al*., 2009; Montoro-García *et al*., 2010), where ammonium sulfate was also the selected precipitant.

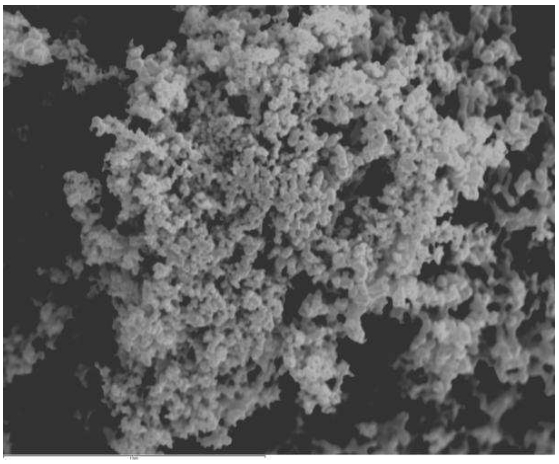
In order to improve not only this low amount of recovered activity but also the stability of the above AS-CLEAs, sodium borohydride (1-10 mg/mL, SB) and BSA (1-10 mg/ml) were used. The use of SB resulted in a 16% increase in activity, reaching 56% (SB-CLEAs), when the optimal SB concentration was used (1 mg/mL) (Table 4.1, vortex column). In addition, co-aggregation of LpNAL with BSA followed by SB treatment (BSA-CLEAs) led to a 2-fold increase in activity, which reached 79% (Table 4.1, vortex column), which represents almost a 30% increase compared with the data obtained with *E. coli* NAL during its immobilization as crosslinked inclusion body (CLIBs) (Nahálka *et al.*, 2008). This increase in activity

Table 4.1 Activity recovery of the CLEAs as a function of mixing period and the system used.

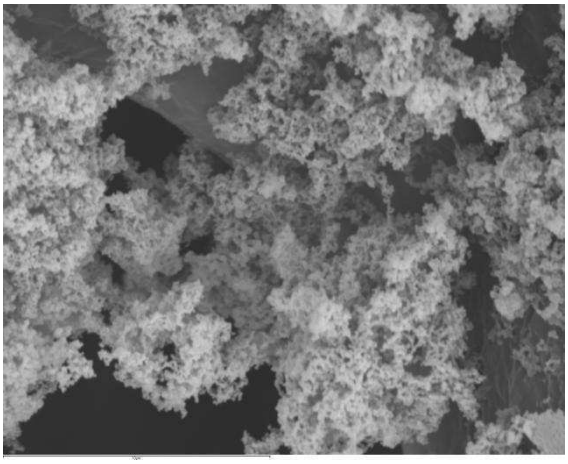
		Vortex		**FastPrep**	
		Mixing period (min.)		Mixing period (min.)	
		0.5[a]	60[a]	0.5[b]	1[b]
Activity recovery (%)	AS-CLEAs	22± 2	40± 1	67± 1	74± 2
	SB-CLEAs	44± 1	56± 3	71 ± 2	72± 1
	BSA-CLEAs	60± 2	79± 1	70± 3	82± 1

In all case the concentration of LpNAL was 0.1 mg/mL.
[a] mixing was carried out with a vortex, at medium speed.
[b] mixing was carried out with FastPrep, at a speed of 6.0 m/s.

is similar to that described for aminoacylase, when BSA was chosen to increase the content of lysine residues and the consequent cross-linking efficiency (Dong *et al.*, 2010). However, in the case of LpNAL, the beneficial effect of the BSA seems to be due to the protective effect of the catalytic residue (lysine) rather than the lack of lysines, as has been described for nitrilase (Mateo *et al*., 2004) and penicillin acylase (Mateo *et al*., 2004). In fact, LpNAL has 24 lysines (7.7% of the protein resides) equally distributed through its sequence, so, the additional 66 lysines contributed by BSA basically prevents any extensive intramolecular crosslinking. The latter benefit of BSA contrasts with the fall in activity described for laccases in the presence of BSA (Cabana *et al.*, 2007; Matijosyte *et al.*, 2010), which was explained by mass transfer limitations (Cabana *et al.*, 2007). In order to test whether such limitations occurred in the three different LpNAL CLEAs described in this chapter, they were subjected to different mixing periods in two disaggregating devices (vortex and FastPrep). Table 4.1 shows that the use of FastPrep, first described by our group in the production of CLEAs (Montoro-García *et al.*, 2010), was the best disaggregating system for all three CLEAs used, not only because of the short time required to reach maximal activity, but also due to the high level of recovery achieved. This FastPrep effect was more evident when a less sophisticated methodology was used to produce the aggregates. The fact that AS-CLEAs activity increased by 34% with FastPrep, compared with the 3% increase displayed by BSA-CLEAs, indicates the greater flexibility of the latter CLEAs resulting form the use of BSA as a proteic feeder (Shah *et al.*, 2006) (Figure 4.1). Planimetric analysis of the different aggregates also showed the influence of the time and dispersing device used (Figure 4.2).



a)



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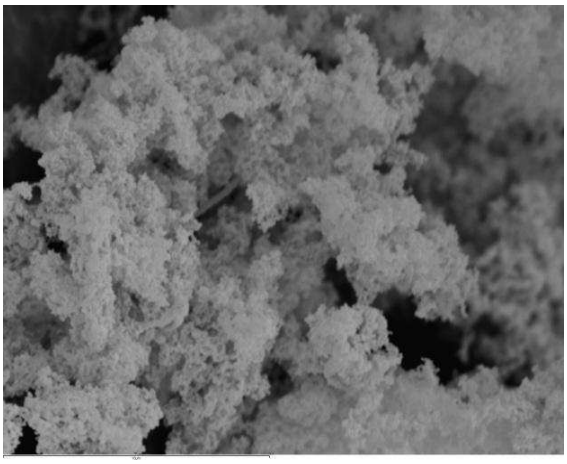

c)

Figure 4.1: *Scanning electron micrographs of FastPrep-CLEAs.* (a) AS-CLEAs, (b) SB-CLEAs and (c) BSA-CLEAs. (Magnification 5500X).

All mechanical treatments fragmented the CLEAs into particles, but most of the aggregates after 30-s vortexing (Figure 4.2, black bars), were distributed in particle sizes up to 1000 μm^2, while 11% were larges (reaching 5000 μm^2). After 1-h vortexing (Figure 4.2, light grey bars), 60% of particles were in the 50-500 μm^2 range, whereas after 60-s of FastPrep treatment (Figure 4.2, dark grey bars), 86% of particles were in the smallest range (0- 50 μm^2).

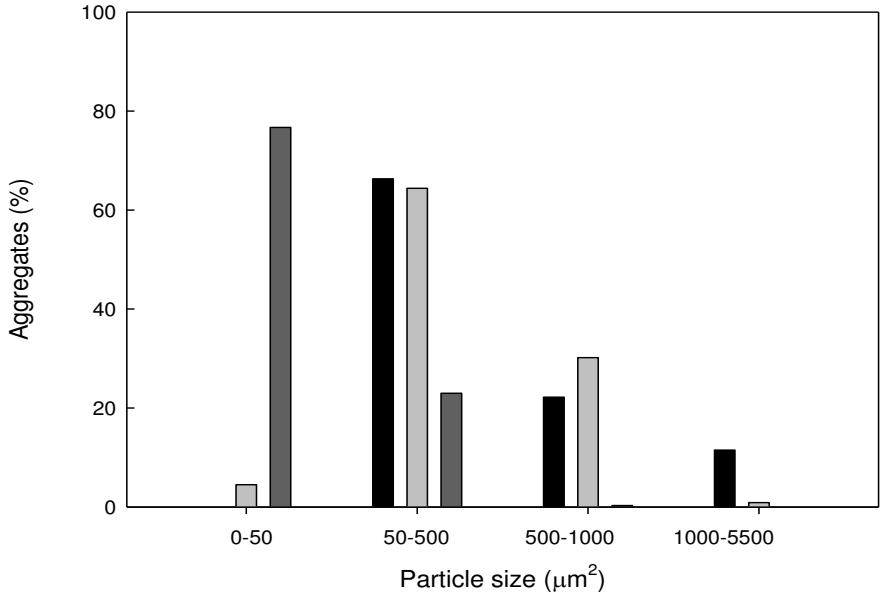

Figure 4.2: *Particle size of CLEAs after different stirring periods*. Black and light grey represent a vortex period of 30 s and 60 min, respectively. Dark grey represents FastPrep precession for 30 s at 6 m/s.

The last treatment, which is also the one that yielded the maximal recovery of the activity, clearly does not disturb the individual CLEAs, and hence, the enzyme structure (as is evident from the SEM analysis, Figure 4.1), preventing that particle

from being compressed close together with concomitant mass-transport limitations. Similar results with Fast-Prep were also reported for acetyl xylan esterase CLEAs, although the particle size obtained was slightly greater, in the 0-149 μm^2 range (Montoro-García *et al.*, 2010). Thus, it is clear from Table 4.1 and Figure 4.2 that the FastPrep system could be of general application to produce efficient CLEAs, regardless of the protocol used to prepare them, and only CLEAs produced with this system were used in the rest of the present work.

4.2.2 Structure of FastPrep-CLEAs

Scanning electron microscopy (SEM) of the FastPrep-CLEAs produced in this paper revealed amorphous structures which differed depending on the protocol used. Thus, the CLEAs obtained with ammonium sulfate (AS-CLEAs) formed less structured and more coarse-grained aggregates (Figure 4.1,a) than those formed by ammonium sulfate and sodium borohydride (SB-CLEAs) (Figure 4.1,b), whereas in the presence of BSA (BSA-CLEAs), they were organized as a network of `branches´ separated by pores, with a spongy and `holey´ morphology (Figure 4.1,c), which maximizes the catalyst surface available for reaction, while minimizing the diffusion effect within the catalyst, as has been previously described for hydroxynitrile lyase (Cabirol *et al*., 2008). These images clearly show the transition from the "classical chemical aggregates" of AS-CLEAs, previously reported in the literature (Tyagi *et al.*, 1999), to a uniform porous mesh (BSA-CLEAs), which is somewhat between the ball-like structure appearance (type 1) and the less structured form (type 2) described by Schoevaart *et al.* (2004). These closely-related type 2 CLEAs of Figure 4.1,c are produced in aggregates with a

hydrophilic nature (Schoevaart *et al.*, 2004), such as those containing LpNAL and BSA, which both have a clear negative Grand average of hydropathicity (GRAVY) number (-0.301 and -0.429, respectively).

4.2.3 Biochemical characterization of free enzyme and FastPrep-CLEAs

The synthetic activity of LpNAL in free form was pH-dependent, being active over a broad pH range, from pH 5.0 to 9.0 (Figure 4.3). The optimum pH of the free enzyme was around pH 7-7.3, which is similar to values described for other NALs, such as those from *E.coli* K1 and C600 (Aisaka *et al.,* 1991; Ferrero *et al.,* 1996), the native and the recombinant NAL from *C. perfringens* (Kruger *et al*., 2001; Nees *et al*., 1976) and the recombinant NAL from *P. multocida* (Li *et al*., 2008). Interestingly, free LpNAL maintained 5-10 % activity in the synthetic direction above pH 11.0, when glycine was used as a buffer. The synthetic activity of LpNAL above pH 10 is a remarkable feature of this NAL compared with those of *E. coli* and *P. multocida* (Li *et al*., 2008) and combats one of industrially relevant drawbacks of this aldolase for the chemo-enzymatic synthesis of Neu5Ac from GlcNAc (Blayer *et al*., 1996). In addition, the enzyme was also stable at basic pHs, where it maintained around 80 % residual synthetic activity after 15 days incubation (Figure 4.3b). The residual activity after incubation at pH 11 was also close to 60 %. This fact, together with the above mentioned higher activity at alkaline pH, reinforce the possibility of using LpNAL to produce Neu5Ac at basic pHs, where the chemical isomerization of GlcNAc to ManNAc is more favorable (Blayer *et al*., 1996; Blayer et al., 1999; Mahmoudian *et al*., 1997).

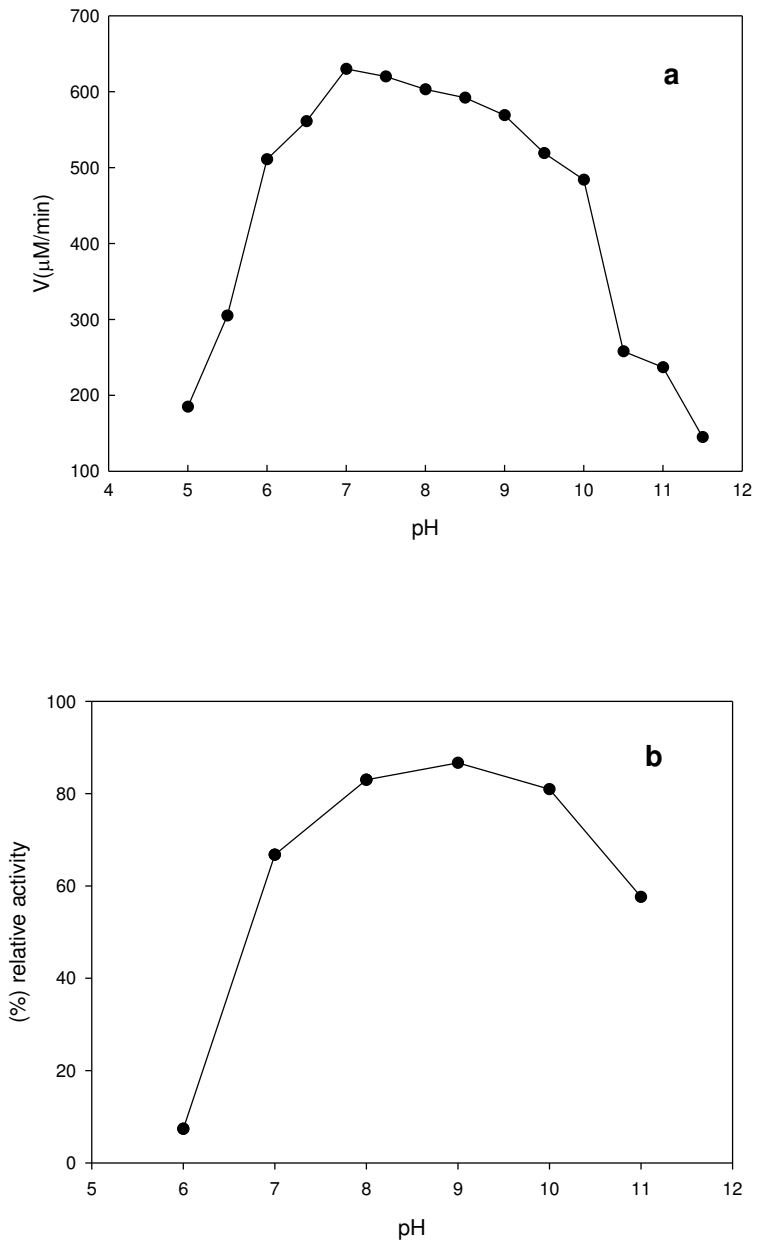

Figure 4.3: *Effect of pH toward LpNAL in free form activity. a) pH profile for the Neu5Ac synthesis activity of LpNAL determined by HPLC*. Assay conditions at 37 ºC were 100 mM N-acetyl-D-mannosamine, 30 mM pyruvate and 350 μg enzyme. The buffers (20 mM) used were sodium acetate pH 5-5.5, sodium phosphate pH 6-8, Tris-HCl pH 8.5-9 and Glycine pH 9.5-11.5. b) pH stability profile in the synthetic direction of LpNAL in free form. Samples were analyzed after 15 days incubation in the different pH media to determine residual activity under the standard reaction, using 100 mM N-acetyl-D-mannosamine and 30 mM pyruvate as substrates. Buffers used were the same as above.

The effect of pH on the catalytic activity of the three different FastPrep-CLEAs produced in this chapter was compared whit that of free enzyme described above. All the CLEAs exhibited a broad bell-shape pH dependence of the catalytic activity, similar to that seen for free enzyme (Figure 4.4a) but with a pH shift towards alkaline, with an optimum of pH 7.5 in contrast to the pH 7.0 of free enzyme. This shift could have resulted from the change in acidic and basic amino acid side chain ionization in the microenvironment around the active site, which was produced by the freshly produced interaction between basic residues of the enzyme and glutaraldehyde during cross-linking, as has been previously described for tyrosinase (Ayart & Bakir, 2008), subtilin (Sangeetha & Abraham, 2008) and acetyl xylan esterase (Montoro García *et al.*, 2010). In addition, the stabilizing effect of BSA (Figure 4.4a, open squares) and sodium borohydride (Figure 4.4a, filled squares) compared with ammonium sulfate alone (Figure 4.4a, open circles) was more evident at alkaline pHs, giving rise to a fully active catalyst at pH 9.0, which is one of the demands for the chemoenzymatic synthesis of Neu5Ac from the inexpensive N-acetyl-D-glucosamine (Liese *et al.*, 2006). In addition, the biocatalyst has to be stable at such alkaline pH values, as is seen from Figure 4.4b, where it is clear that only BSA-CLEAs (Figure 4.4b, open squares) were as stable at pH 9.0 as the free enzyme (Figure 4.4b, filled circles), and more stable than the SB-CLEAs (Figure 4.4b, filled squares). The standard AS-CLEAs were clearly not stable at such pHs (Figure 4.4b, open circles).

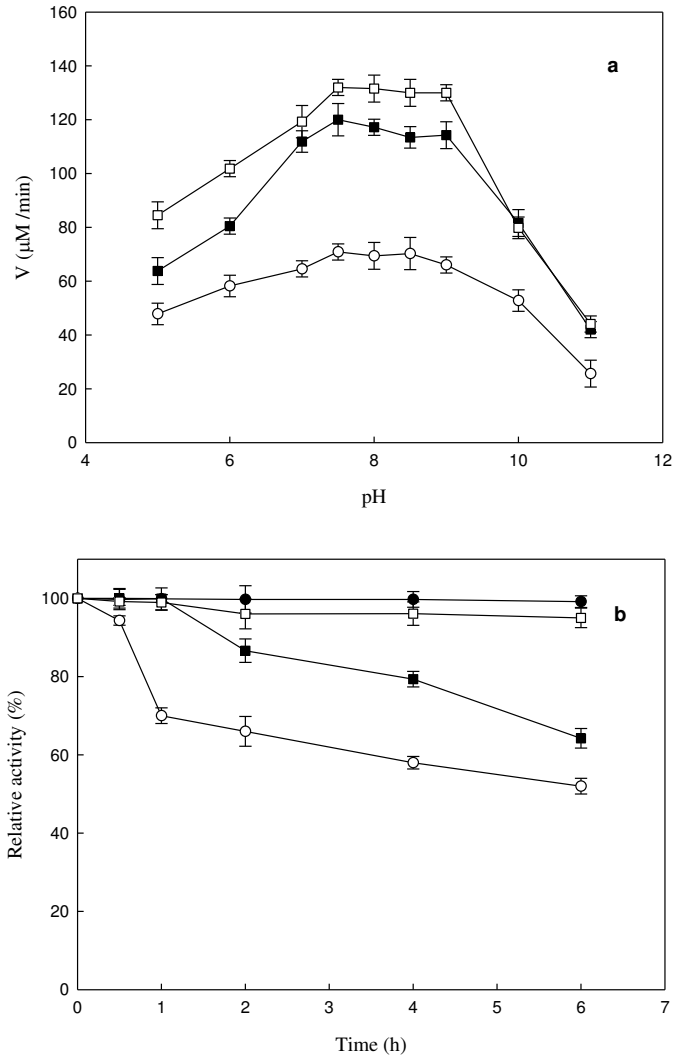

Figure 4.4: *Effect of pH on different FastPrep-CLEAs.* (a) pH profile for the Neu5Ac synthesis activity of AS-CLEAs (○), SB-CLEAs (■), BSA-CLEAs (□) determined by HPLC under standard conditions at 37 ºC. The buffers (20 mM) used were sodium acetate pH 5-5.5, sodium phosphate pH 6-8, Tris-HCl pH 8.5-9 and Glycine pH 9.5-11.5. (b) Stability at pH 9.0 and 50 ºC of free enzyme (●) and different FastPrep-CLEAs produced: AS-CLEAs (○), SB-CLEAs (■), BSA-CLEAs (□). Residual activity was studied in 20 mM glycine buffer pH 9 using 100 mM ManNAc and 30 mM pyruvate as substrates and 0.1 mg/mL of enzyme.

The optimal temperature of free LpNAL was measured in synthetic direction by HPLC, showing an optimal temperature of 60 ºC for synthetic (Figure 4.5), temperature far from the optimal growth temperature of *Lactobacillus plantarum* WCFS1, which is 30 ºC. These values are similar to these described for EcNAL (~80 ºC) (Aisaka *et al.,* 1991), although all the kinetic parameters were determined at physiological temperatures (30-37 ºC) for all the NALs reported (Aisaka *et al.,* 1991; Li *et al*., 2008). No thermal stability improvements between free and BSA-CLEAS due to cross-linking were observed, since NALs are very thermostable proteins with melting points (T_m) of about 74 ºC at pH 9.0 (Sánchez Carrón *et al.*, 2010) and an optimal temperature about of 70-80 ºC (Aisaka *et al.*, 1991).

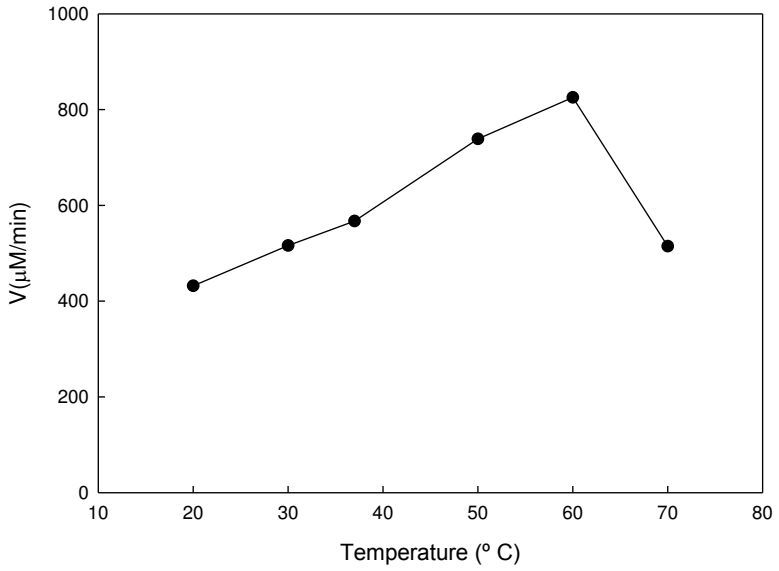

Figure 4.5: *Temperature profile for the Neu5Ac synthesis activity of LpNAL determined by HPLC.* Assay conditions were 100 mM N-acetyl-D-mannosamine, 30 mM pyruvate and 350 µg enzyme in 20 mM sodium phosphate buffer pH 7.

4.2.4 Kinetic parameters native enzyme and CLEAs

The kinetic parameters for the synthetic activity were also investigated with the best FastPrep-CLEAs obtained with BSA and SB (Fp-CLEAs) at different ManNAc and pyruvate concentrations, and compared with free enzyme (Sánchez Carrón *et al.*, 2010). For both substrates, Michaelis-Menten type kinetic behaviour was observed for the CLEAs. As shown in Table 4.2, the apparent K_M for immobilized enzyme was 1.5-fold higher for ManNAc than for free enzyme, whereas it was 2.2-fold lower for pyruvate. This differential change in affinity has also been described for the affinity of acetyl xylan esterase for 7-ACA and cephalosporin-C (Montoro-García *et al.*, 2010), but there seem to be no clear rule, since both increases (Shah *et al.*, 2006; Montoro-García *et al.*, 2010) and decreases (Aytar & Bakir, 2008; Sangeetha & Abraham, 2008) in K_M values have been observed in different CLEAs. These data (Table 4.2) indicate that the interaction of enzyme and substrate was stronger in the case of pyruvate, and possibly, that molecular hindrances are involved in the case of ManNAc after cross-linking with glutaraldehyde. The latter reagent was also responsible for the decrease in the turnover and in the catalytic efficiency (k_{cat}/K_M) of the enzyme for both substrates (Table 4.2), clearly indicating an attack on the catalytic lysine of NAL. Nevertheless, and in spite of the values shown in Table 4.2, these CLEAs are of biotechnological relevance. The apparent K_M for ManNAc is lower than that reported for the enzymatic synthesis of Neu5Ac, with a K_M values of 402 mM (Kragl *et al.*, 1991), which needs concentrations about 0.7 M to reach maximum activity. On the other hand, the lower K_M of pyruvate is

also advantageous, since a high pyruvate concentration in the solution after the bioconversion hinders product recovery, due to the fact that pyruvate and Neu5Ac exhibit similar pKa values in the region of 2.2 (Kragl *et al*., 1991), making a tedious ion-change chromatography step necessary (Sugai *et al.*, 1995).

Table 4.2 Kinetic parameters of free enzyme and enzymes in Fp-CLEAs.

	K_M (mM)		k_{cat}/K_M ($mM^{-1}\cdot s^{-1}$)	
	Soluble enzyme	Fp-CLEAs¶	Soluble enzyme	Fp-CLEAs
ManNAc	160± 5	245± 5	0.03	0.0074
Pyr	19.9± 0.3	9± 1	0.11	0.09

¶ Activity was assayed as described in Materials and Methods, at 37ºC and using 0.1 mg/mL of the enzyme.

4.2.5 Potential production of Neu5Ac

To investigate the potential of LpNAL CLEAs for the production of the Neu5Ac in aqueous medium, the condensation of ManNAc and pyruvate by Fp-CLEAs was studied. In this enzymatic reaction, an excess of pyruvate over ManNAc is generally used to achieve high yield of Neu5Ac, because the equilibrium tends towards pyruvate and ManNAc (K_{eq}, 28.7 M^{-1} at 25 ºC) (Kragl *et al*., 1991). For this reason,

the Fp-CLEAs reaction was carried out at 30 ºC with an almost three-fold molar excess of pyruvate over ManNAc (1.4M/0.5M) to archive a high reaction rate. When the reaction was approaching its equilibrium point, after about 13 hours (Figure 4.6, open circles), the reaction temperature was reduced to 20 ºC (Figure 4.6, open diamonds) to obtain 77% conversion. Figure 4.6 also shows the comparison of the temperature shifting reaction with another reaction in which pyruvate (0.35 M) was fed three times after the equilibrium point was achieved at 5, 10 and 13 hours (Figure 4.6, arrows), respectively. In both cases, the same yield was obtained, indicating the advantage of the temperature shifting approach due to its simplicity and no need for repetitive additions of pyruvate.

In addition, the yield obtained with Fp-CLEAs from ManNAc was higher than that obtained with commercial Neu5Ac aldolase (60%) (Sugai *et al.*, 1995), but lower than that obtained with a 10-fold higher concentration of enzyme in a free form (Blayer *et al.*, 1996), which clearly avoids the possibility of enzyme inactivation produced during longer periods of reaction, as a result of pyruvate degradation (Blayer *et al.*, 1996).

Having demonstrated Neu5Ac production with LpNAL Fp-CLEAs, their stability during multiple reuses was investigated, using the temperature shifting approach. Typically, reactions (1 mL) reached completion within 15 hours, and at least 10 cycles were possible without any significant loss of activity (Figure 4.6, inset). Such stability is the same as that observed when Neu5Ac aldolase is used in an industrial process, with a known solid support like Eupergit-C (Mahmoudian *et al.*, 1997) or when it is immobilized into cross-linked inclusion bodies (CLIBs) (Nahálka *et al.*, 2008). This strongly supports the value of using hardened CLEAs in

combination with the FastPrep system for dispersion, to yield Fp-CLEAs with excellent resistance to mechanical stress, allowing high recoveries of catalyst after many cycles of use. These ten cycles without loss of activity in CLEAs has also been described for lipase (Gupta *et al.*, 2009), R-oxynitrilase (van Langen *et al.*, 2004) and acetyl xylan esterase (Montoro-García *et al.*, 2010), but constrast with the four reuses possible with hydroxynitrile lyase (Cabirol *et al.*, 2008) or subtilisin (Sangeetha and Abraham, 2008).

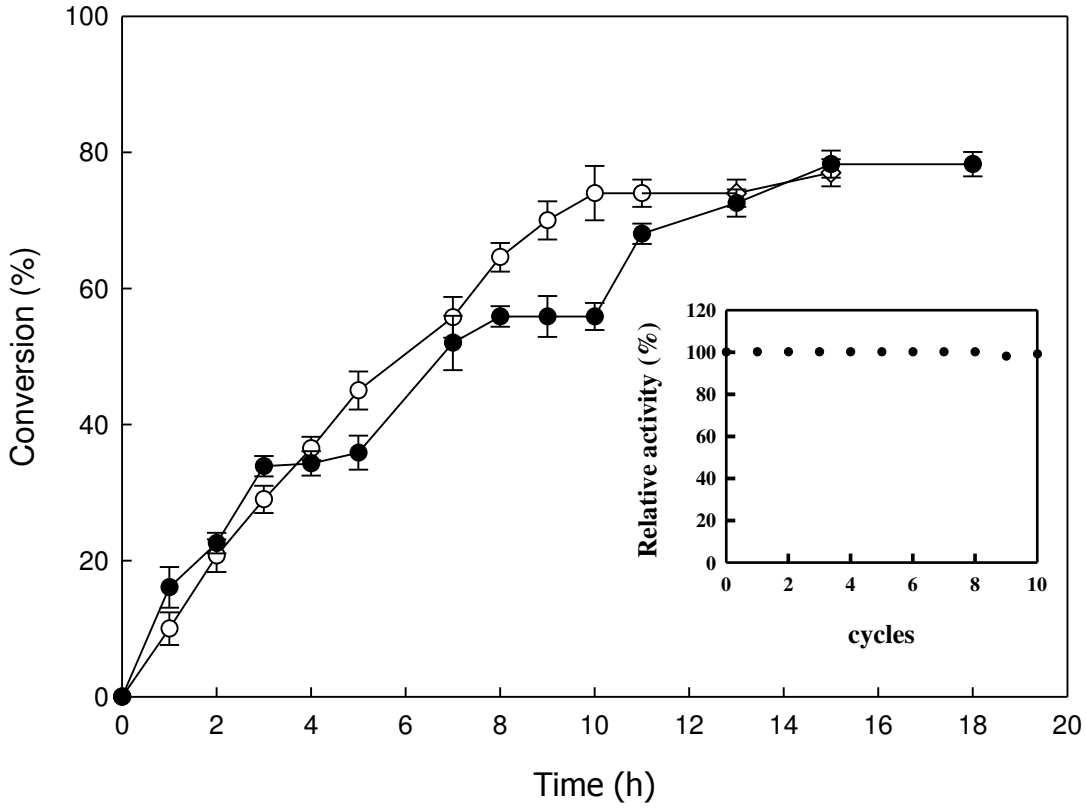

Figure 4.6: *Time-course of Neu5Ac synthesis from ManNAc and pyruvate using Fp-CLEAs* (○). Temperature-shifting approach. Reaction conditions at 30 ºC were 0.5 M ManNAc, 1.4 M pyruvate and 0.1 mg/mL Fp-CLEAs in 20 mM phosphate buffer pH 7.5. After 13 hours, the temperature was decreased to 20 ºC (◊). (●) Pyruvate feeding approach. The reaction was carried out at 30 ºC in 20 mM phosphate buffer pH 7.5 containing 0.1 mg/mL Fp-CLEAs, 0.5 M ManNAc and 0.35 mM

pyruvate. Arrows showed the feeding points with 0.35 M sodium pyruvate. The amount of Neu5Ac in the reaction mixture was determined by HPLC-ELSD (see Materials and Methods). *Inset*: *Operational stability of CLEAs in the biotransformation of ManNAc and pyruvate in a 1mL reactor*. After 15 hours reaction, Fp-CLEAs (0.l mg/mL) were centrifuged, the pellet was resuspended in a new reaction solution containing 0.5 M ManNAc and 1.4 M pyruvate, in 20 mM phosphate buffer pH 7.5 and the activity was measured.

4.3 CONCLUSIONS

The results presented here highlight the potential of using hardened CLEAs (BSA and sodium borohydride) for obtaining an advanced intermediate for the antiviral Relenza®, such as Neu5Ac. The combinations of such robust CLEAs with an efficient dispersing methodology (FastPrep) reduces to 30 s the time needed to produce highly active Fp-CLEAs with strong operational stability at alkaline pHs, where the industrial enzymatic process is carried out. These findings open up new pathway for using Fp-CLEAs for producing Neu5Ac.

Chapter V

Characterization of a novel N-acetylneuraminate lyase from *Staphylococcus carnosus TM300* and its application to N-acetylneuraminic acid production.

5.1 INTRODUCTION

N-acetylneuraminic acid (Neu5Ac) is a key monosaccharide unit in brain gangliosides and glycoproteins, including polyN-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 (Kiss & Rougon, 1997; Rutishauser & Landmesser, 1996). 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 (Wang *et al.*, 1998). Importantly, the concentration of Neu5Ac in the frontal cortex of breast-fed infants is higher than in formula-fed infants (Wang *et al*., 2003). 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 (Wang *et al*., 2003). 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 (Wang *et al*., 2001). 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 to preparing advanced intermediates for antivirals, such as Relenza® (GSK) (Liese *et al*., 2006; Itzstein, 2007; García-García *et al*., 2011).

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 favours 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 (Huang *et al*., 2007; Yu *et al*., 2004). However, most NALs described to date come from human pathogens, except for the NAL cloned from the commensal *Lactobacillus plantarum* (Sanchez-Carrón *et al*., 2011). This enzyme is crucial to the species concerned 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, since it plays a central role in the generation of the overall flavor of fermented sausages. Indeed, it has a long history of safe use in many fermented sausages and meat.

This Chapter describes its cloning and characterization from the *nanA* gene of *Staphylococcus carnosus* TM300. Its high expression, easy purification procedure (with the highest recovery achieved in the bibliography), 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 production costs of Neu5Ac for use in the food industry.

5.2 RESULTS AND DISCUSSION

5.2.1 Amino acid sequence analysis

The deduced amino acid sequence of the *S. carnosus N*-acetylneuraminate lyase (ScNAL) showed significant identity with the sequences of other bacterial species in the database. Sequence alignment indicates that ScNAL has 58%, 56%, 48% and 35% amino acid sequence identity with the *N*-acetylneuraminate lyase from *Haemophilus influenzae, Clostridium perfringens, Lactobacillus plantarum* and *E. coli* K-12 (GenBank accessions: Q9S4K9, P44539, CAD65647.1 and AAC76257), 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 (Combet *et al*., 2002). In addition, sequence alignment (Figure 5.1) revealed that ScNAL contained the conserved residues forming the characteristic active site of the NAL subfamily (Figure 5.1, filled triangles): 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 (Barbosa *et al*., 2000).

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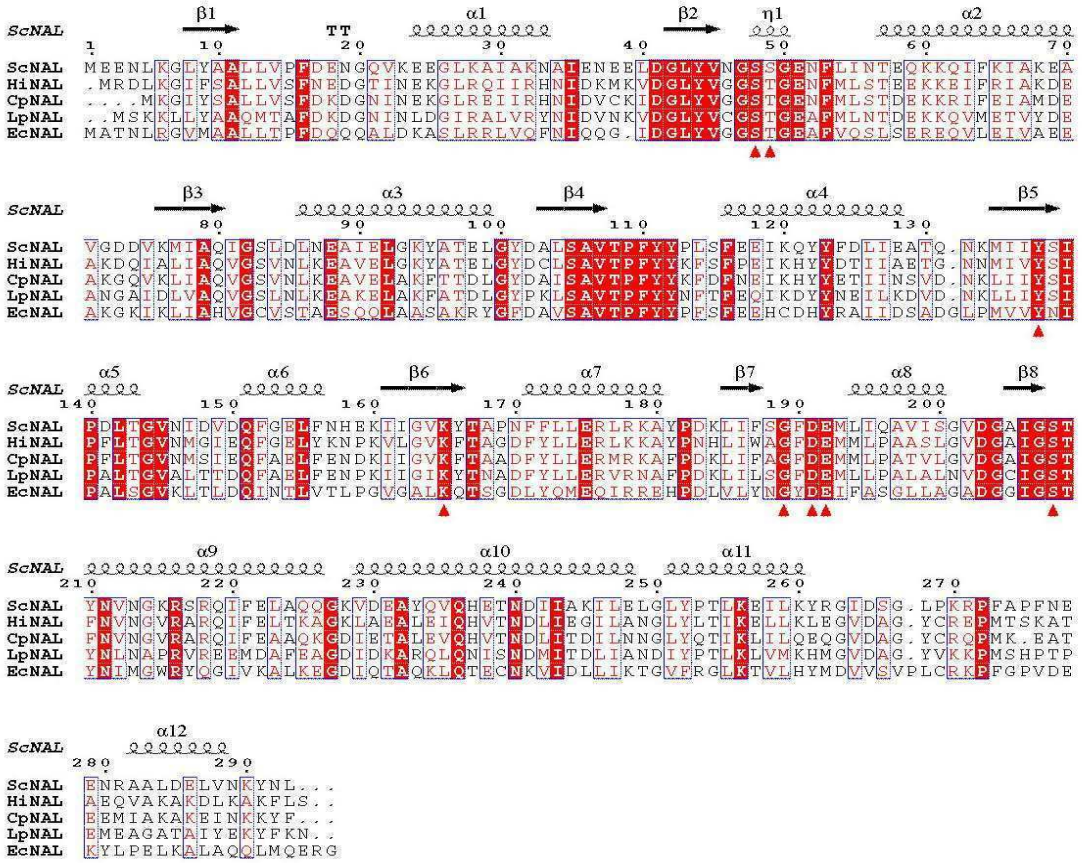

Figure 5.1: *Multiple sequence alignment for* Staphylococcus carnosus *TM300 (ScNAL) and related N-acetylneuraminate lyases*. ESPript output (Gouet *et al*., 1999).was obtained with the sequences from the NCBI and aligned with CLUSTAL-W (Thompson *et al*., 1994). Sequences were grouped according to similarity. 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 red 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 small red triangles.

5.2.2 Overexpression and purification of ScNAL

Staphylococcus carnosus TM300 *nanA* gene was cloned into pET28a vector which provides His_6-tagged recombinant protein (see Materials and methods). The DNA sequence of the inserted gene showed no mutations compared with 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 ºC for 12 h with vigorous stirring and oxygenation. 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 onto a HisTrap FF column. After these two steps, the enzyme was pure, as shown in SDS-PAGE (Figure 5.2, lane 3) with a 7-fold purification (Table 5.1).

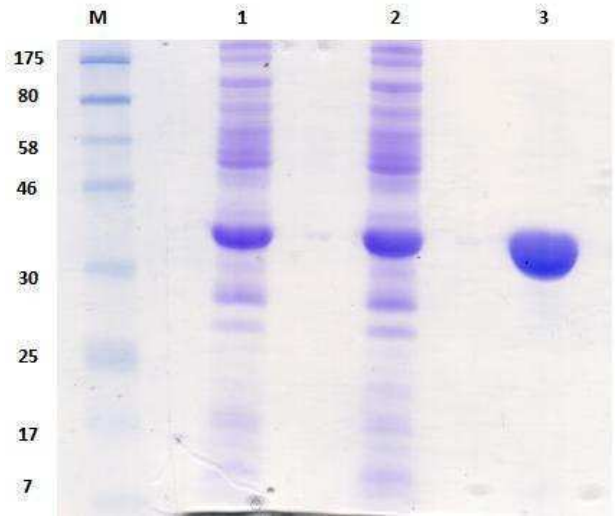

Figure 5.2: *SDS-PAGE of the ScNAL gene product obtained after 12 hours IPTG induction*. Each lane contained 15 µg of protein. M: molecular weight standards (New England Biolabs: P7708S). Lane 1: cell extract after DNase treatment. Lane 2: Cell extract after 50-kDa tangential ultrafiltration. Lane 3: ScNAL after HisTrap column (purified protein is about 35 kDa).

This two-step purification procedure represents a significant improvement over other methods described for NAL, since 86 % recovery was achieved (Table 5.1),

which is a 2- to 4-fold higher than the recovery described for LpNAL, whose purification is performed in three steps (42.3% recovery) (Sanchez-Carrón *et al*., 2011) and for *Clostridium perfringens* aldolase, which needs 5 purification steps (21% recovery) (DeVries & Binkley, 1972). ScNAL, thus obtained, showed a specific activity of 12.7 U/mg for the hydrolysis of Neu5Ac at 37 ºC and pH 7.0 (Table 5.1). 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 two-fold increase compared with the best expression level reported for LpNAL (215 mg L^{-1}) (Sanchez-Carrón *et al*., 2011) and 4–time higher than others NALs (Yu *et al*., 2004).. The molecular weight of purified protein was determined by gel filtration (126.40 kDa), by HPLC/MS/ESI (37.33 kDa) and by 12% SDS-PAGE (35 kDa) (Figure 5.2, lane 3), confirming the homotetrameric nature of ScNAL.

Tabla 5.1 Purification of recombinant ScNAL

Purification Step	**Volume (ml)**	**Total Activity¶ (U)**	**Total protein (mg)**	**Specific activity (U/mg)**	**Purification (*x*-fold)**	**Yield (%)**
Crude extract†	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

¶The activity was assayed in the standard spectrophotometric reaction medium.

†Crude extract represents the volume obtained after lysis, centrifugation of cell debris and DNase treatment corresponding to a 1 L culture broth. See Material and Methods for details.

5.2.3 Biochemical characterization of recombinant ScNAL

The activity of ScNAL was pH-dependent, and was active over a broad pH range in both synthetic (Figure 5.3a, squares) and hydrolytic directions (Figure 5.3a, circles). 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 (Aisaka, et al., 1991; Ferrero *et al*., 1996), the native and the recombinant NAL from *C. perfringens* (Kruger *et al*., 2001), the recombinant NAL from *P. multocida* (Li *et al*., 2008) and the recombinant NAL from *L. plantarum* WCFS1 (Sánchez-Carrón *et al*., 2011).

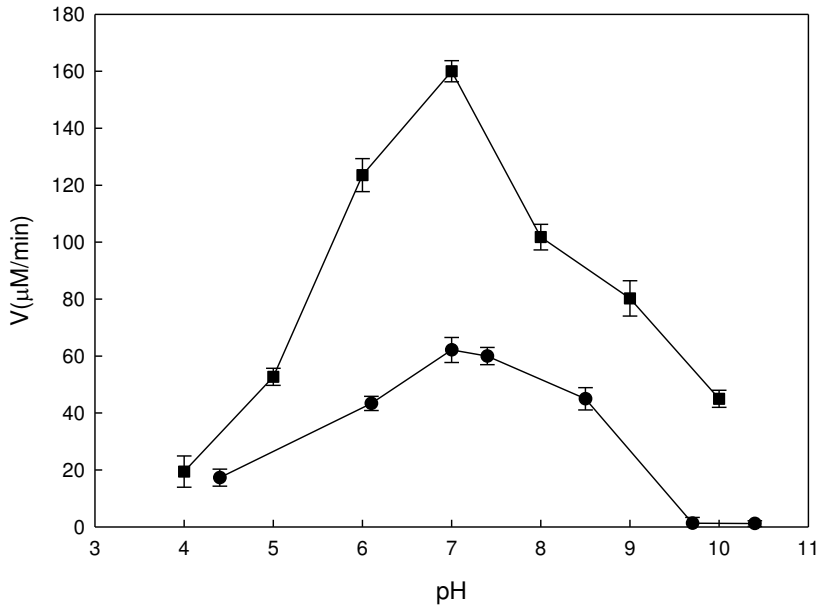

Figure 5.3: *Effect of pH on ScNAL activity*. a) pH profile for Neu5Ac synthesis (■) and hydrolysis (●) determined by HPLC. Assay conditions at 37 ºC were 100 mM N-acetyl-D-Manosamine, 30 mM pyruvate and 20 μg enzyme for the synthetic assay, and 10 mM Neu5Ac and 4 μg enzyme for the hydrolytic assay. 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.

ScNAL showed 50 % activity in the synthetic direction above pH 9.0, when glycine was used as a buffer (Figure 5.3). In addition, the enzyme was also stable at basic pHs, 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 5.4). ScNAL was less stable at such basic pHs, compared with other previously studied NALs, such as EcNAL, which maintains 70% of its activity after 8 hours at pH 10.5 (Aisaka & Uwajima, 1986) or *Lactobacillus plantarum* NAL, which maintains around 80% residual synthetic activity after 15 days of incubation at pH 9 (Sanchez-Carrón *et al*., 2011).

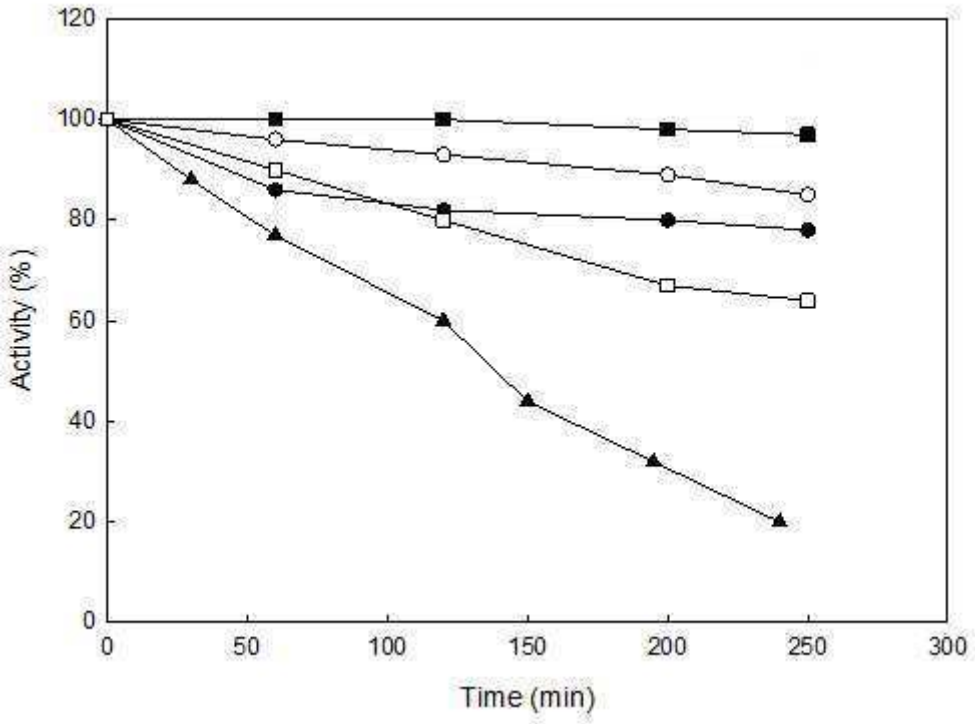

Figure 5.4: *pH-stability profiles of the purified ScNAL at 37 ºC*. The enzyme was incubated for different periods of time at pH 5 (□) pH 6 (○) pH 7 (■), pH 8 (●) and pH 9 (▲), and the activity was measured by HPLC under the standard reaction conditions at pH 7, using ManNAc and pyruvate as substrates.

The optimal temperature of ScNAL was measured by HPLC, and was found to be 50 °C for synthetic activity (Figure 5.5, squares) and of 60-70 °C for the hydrolytic reaction (Figure 5.5, circles), which is somewhat lower than that described for EcNAL (80 °C) (Aisaka *et al*., 1991) and similar to LpNAL (70 ºC) (Sanchez-Carrón *et al*., 2011). 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 (Lee *et al*., 2004), which affects the kinetic constants via the Haldane relationship (Zimmerman *et al*., 2007). Both temperatures are far from the optimal growth temperature of *Staphylococcus carnosus* TM 300, 30 to 37 °C, the range in which the kinetic parameters of NAL are usually determined (Aisaka *et al*., 1991; Li *et al*., 2008; Sánchez-Carrón *et al*., 2011).

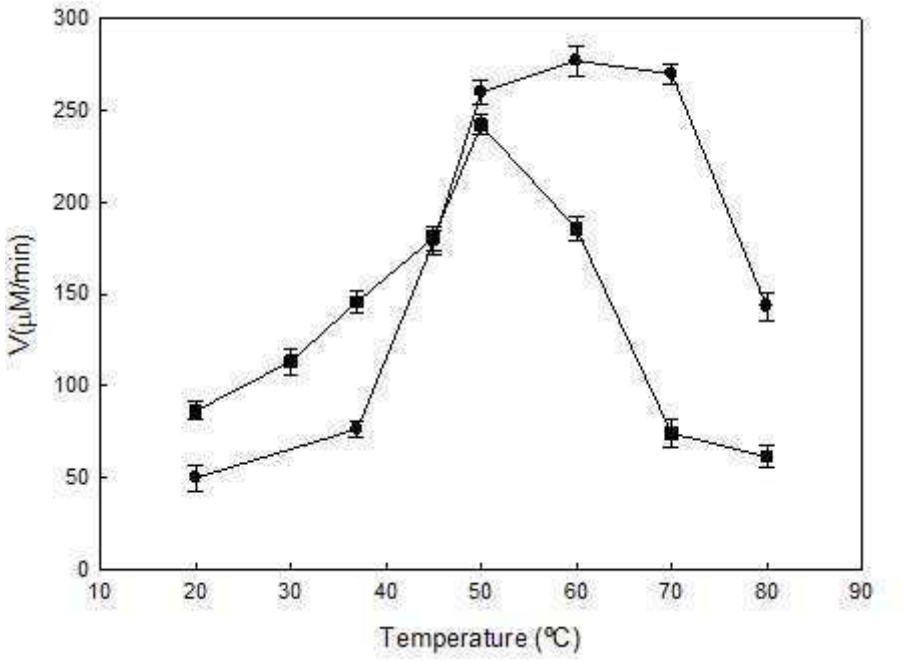

Figure 5.5: *Effect of temperature on ScNAL activity*. a) Temperature profile for Neu5Ac synthesis (■) and hydrolysis (●). Conditions were the same as Figure 3, but at different temperatures. Independent experiments were conducted with a minimum of three replicates per condition.

Thermal stability was also determined, in a temperature range of 60 to 80 °C (Figure 5.6), showing that ScNAL was most stable at temperatures between 60 and 70 ºC, but strongly decreased its stability at temperatures above 80 °C.

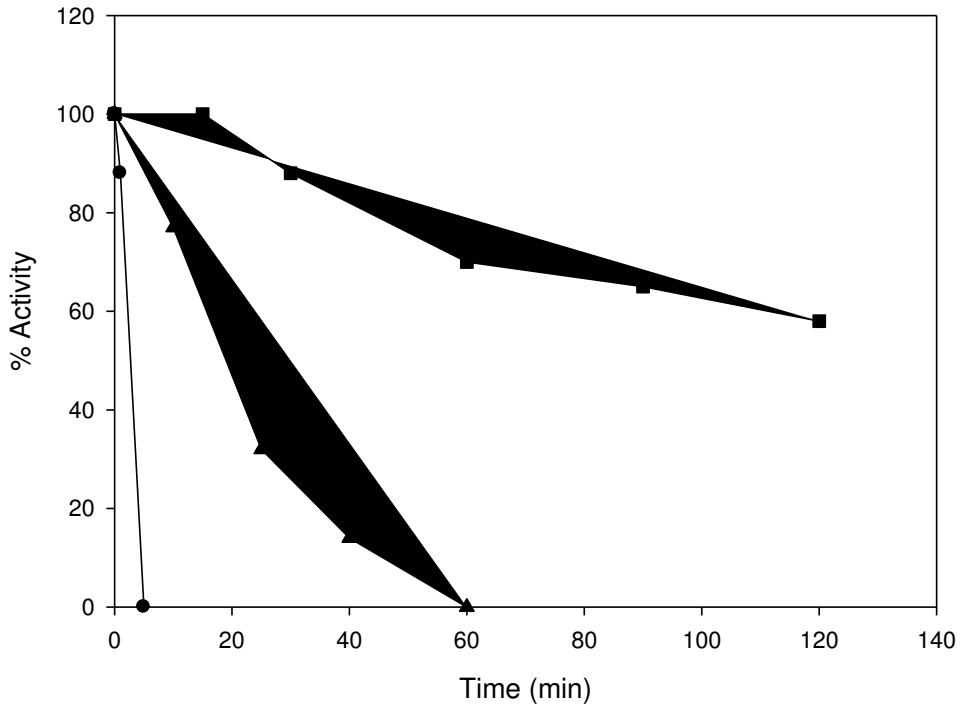

Figure. 5.6 *Temperature stability profiles of the purified ScNAL at pH 7.0*. The enzyme was incubated for different periods of time at 60 °C (■), 70 °C (▲), and 80 °C (●), and then, the activity was measured spectrophotometrically, under the standard reaction conditions at 37 °C using Neu5Ac as the substrate.

This thermal stability was further confirmed by a thermal melt assay, a T_m value of 62±0.2 ºC being obtained for ScNAL in Milli-Q® water (Figure 5.7, open diamonds). The presence of a buffer solution (100 mM sodium phosphate pH 7.0) stabilized the enzyme, raising the T_m value to 66±0.2 ºC (Figure 5.7, closed diamonds).

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 ºC, 80±0.1 ºC and 82±0.2 ºC, respectively (Figure 5.7). In addition, ScNAL showed great thermal stability from pH 5 to 7.8 with the T_m ranging from 60 to 66 ºC, whereas T_m fell drastically to only 40 ºC above pH 8.0. These data are in agreement with the optimal pH obtained (Figure 5.3) a higher T_m value could indicate that the buffer induces structural changes in the protein to a more ordered conformation, whereas a lower T_m value may be an indication of destabilization (Ericsson *et al*., 2006). These changes are similar to those studied previously for other proteins, such as LpNAL (Sánchez-Carrón *et al*., 2011) or anthrax protein (Sorrell *et al*., 2010).

In order to study the possible structural changes induced by substrates (ManNAc and pyruvate) and related compounds (D-mannose, glyoxilic 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 °C to 78±0.1 ºC at 80 mM pyruvate (Figure 5.7 inset, see also Figure 5.7, filled circles). These results suggest a direct interaction between pyruvate and the enzyme via the Schiff base, as has been described in crystals of EcNAL with hydroxypyruvate (Lawrence *et al*., 1997).

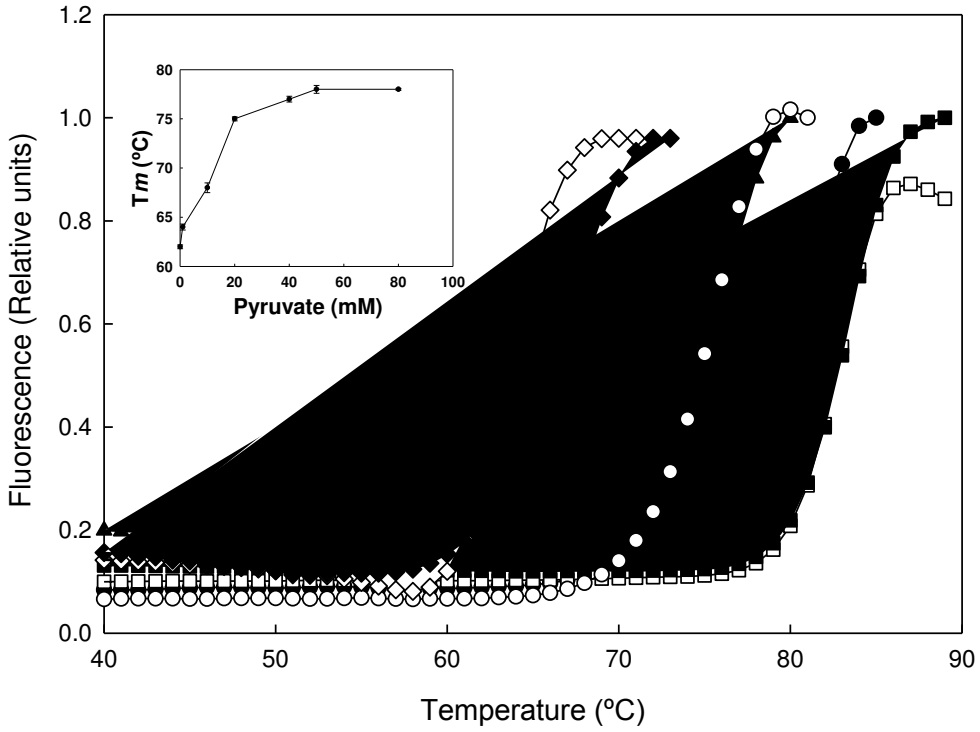

Figure 5.7: *Study of thermal stability of ScNAL*. Melting temperature curves of purified enzyme (1µg) were used in Milli-Q® water (◊), in the presence of 20 mM buffer pH 7 (♦) and in the presence of additives, such as 1 M hydroxy-ectoine (▲), 1 M ammonium sulphate (■), 0.4 M ammonium sulphate (□), 20 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.

5.2.4 Kinetic parameters

The kinetic constants were determined in both cleavage and synthetic directions (Table 5.2). The K_M for Neu5Ac cleavage (2 mM) was lower than the values reported for *E. coli* NAL (2.5-3.8 mM) (Aisaka *et al*., 1991), *P. multocida* (4.9 mM) (Li *et al*., 2008) and *C. perfringens* (2.8-3.2 mM) (Kruger *et al*., 2001) and similar to that of *L. plantarum* (1.8 mM) (Sánchez-Carrón *et al*., 2011). This value, together with the k_{cat}/K_M value obtained (2 mM^{-1} s^{-1}), points to the similar catalytic activity of this new recombinant ScNAL for hydrolysis to that of other previously reported NALs for the same substrate (3.1 to 4 mM^{-1} s^{-1}) (Sánchez-Carrón *et al*., 2011).

On the synthetic side, ScNAL showed K_M values of 149 mM and 14 mM for ManNAc and pyruvate, respectively. These values are also lower than those described for EcNAL (180 mM and 22 mM), PmNAL (220 mM and 23 mM) (Li *et al*., 2008), and LpNAL (160 mM and 19.9 mM) (Sánchez-Carrón *et al*., 2011). As occurs in Neu5Ac hydrolysis, ScNAL is also efficient for the synthesis of Neu5Ac, showing k_{cat}/K_M values of 0.03 mM^{-1} s^{-1} for ManNAc and 0.2 mM^{-1} s^{-1} for pyruvate, which are similar to those previously reported for other NALs (Sánchez-Carrón *et al*., 2011).

Table 5.2 Kinetic parameters of ScNAL for Neu5Ac cleavage and synthesis.¶

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

¶The activity was assayed by HPLC in each standard reaction medium for cleavage and synthesis, respectively (see Material and methods for details).

5.2.5 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 towards pyruvate and ManNAc (K_{eq}, 28.7 M^{-1} at 25 ºC) (Kragl *et al*., 1991). For this reason, the reaction was carried out at 25 ºC with an almost two-fold molar excess of pyruvate over ManNAc (1.05 M *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 the enzyme concentrations used (3-12 U/mL) (Figure 5.8). 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 (Blayer *et al*., 1999), and higher than those obtained with recombinant *E. coli* C600 NAL (Lee *et al*., 2004), using an eight-fold excess of pyruvate over ManNAc. In addition, this conversion is independent of the feeding strategy used, and is the same whether pyruvate is added once or fed three times (0.35 M) after reaching the equilibrium points were reached at 4, 7 and 20 hours, respectively.

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* (2004), who reported that the conversion yield of Neu5Ac increased from 40 % at 50 ºC to 70 % at 20 ºC 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 ºC, as shown in Figure 5.8 (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 ºC) with 12 U/mL ScNAL.

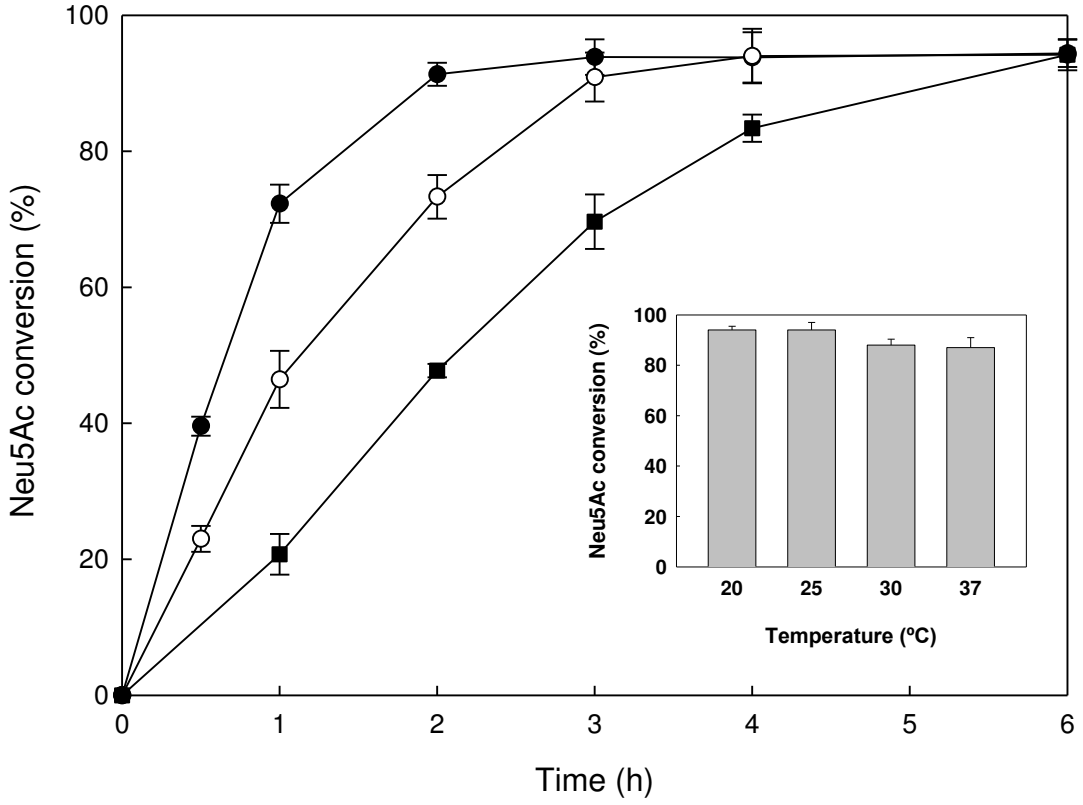

Figure 5.8: *Time course of Neu5Ac synthesis from ManNAc and pyruvate using ScNAL as biocatalyst*. The experiments were carried out at 25 ºC with 0.55 M ManNAc, 1.05 M pyruvate and different ScNAL concentrations: 3 (■), 6 (○) and 12 U/ml (●). Inset. Effect of temperature on Neu5Ac production. Conditions are the same as above (12 U/ml), except for temperature. Independent experiments were conducted with a minimum of three replicates per condition.

5.3 CONCLUSION

This study showed that it is possible to use *Staphylococcus carnosus* NAL to synthesize Neu5Ac, with the same conversion yield, time and units as reported for, the best industrial biocatalyst. However, the aldolase was obtained from a gene of a GRAS microorganism rather than of a one from a pathogenic microorganism and with no problems in scaling up the preparation, due to the high expression and activity of the ScNAL. The above advantages, together with its easy purification and high enzyme recovery, which decrease the cost of biocatalyst, the simple working conditions used (2- fold molar ratio pyr/ManNAc, pH 7.0, no buffer, 25 ºC), the short conversion time (3 hours at 12 U/mL ScNAL), and the known direct crystallization of Neu5Ac from the reaction solutions using glacial acetic acid (Maru *et al*., 2002), make ScNAL a promising biocatalyst for obtaining Neu5Ac for fortified foods, including infant formulas.

CHAPTER 6. Production of KDN with recombinant aldolase immobilized in FpCLEAs.

Contenido inhibido autorizado por la Comisión General de Doctorado de fecha 21 de febrero de 2013.

CHAPTER 7. Molecular characterization of N-acetyl neuraminate lyases from the previously unstudied group 3

Contenido inhibido autorizado por la Comisión General de Doctorado de fecha 21 de febrero de 2013.

CHAPTER 8. Molecular charcaterization of a new N-acetylneuraminate synthase from *Idiomarina loihiensis*.

Contenido inhibido autorizado por la Comisión General de Doctorado de fecha 21 de febrero de 2013.

IX. General Discussion

The enzymes studied in this thesis have been N-acetyl neuraminate lyase (NAL) and N-acetyl neuraminate synthase (NAS), both proteins directly related to the bacterial metabolism of sialic acid. As regard the first, NALs are enzymes that catalyze the hydrolysis of N-acetylneuraminic acid (sialic acid or Neu5Ac) to pyruvate and N-acetyl-D-mannosamine (ManNAc). These enzymes also perform the inverse of the aldol condensation reaction for synthesise the N-acetylneuraminic acid (Neu5Ac) and certain derivatives from pyruvate and N-acetyl-D-mannosamine (ManNAc) (Aisaka *et al*., 1991, Lee *et al*., 2008, Sánchez Carrón *et al*., 2011, García-García *et al*., 2012). In their four different NALs have been cloned and characterized from non-pathogenic microorganisms; *Lactobacillus plantarum* (LpNAL), *Staphylococcus carnosus* (ScNAL), *Lactobacillus antri* (LaNAL) and *Lactobacillus sakei* (LsNAL). In addition, an N-acetylneuraminate synthase, from another non-pathogenic microorganism (*Idiomarina loihiensis*; IlNAL) was studied. Neu5Ac synthase is a metal-dependent enzyme, which produces Neu5Ac from ManNAc and PEP in an irreversible reaction (Suryanti *et al*., 2003; Gunawan *et al*., 2005). In addition, some of these enzymes have been screened as potential biocatalysts with industrial application. For example, LpNAL and ScNAL were immobilized as FpCLEAs for the production of Neu5Ac, and its derivative KDN, respectively.

1. Production and characterization of FastPrep-Stabilized CLEAs of *Lactobacillus plantarum* Neu5Ac aldolase for sialic acid synthesis.

In order to develop a new possible biocatalyst for the possible industrial applications in the synthesis of Neu5Ac, a search of the databases (NCBI and Uniprot) was carried out to find new possible NALs from non-pathogenic microorganisms, the best candidate was *Lactobacillus plantarum* WCFS1 NAL. This

enzyme was cloned and purified, showing a high synthetic activity not only at its optimum pH, but also at basic pHs. This together with its high thermostability and pH-stability at basic pHs, makes LpNAL a promising biocatalyst. In order to explore its potential biotechnological application in the synthesis of sialic acid and its derivatives, the enzyme was immobilized as crosslinked enzyme aggregates (CLEAs), but its loss of activity was minimized by adding stabilizers such as bovine serum albumine and sodium borohydride, while its size was improved by the use of a cell disruptor (Fast-Prep). With these optimized FpCLEAs of LpNAL, a high production of Neu5Ac was achieved (77% conversion) (García García *et al*., 2011). Thus, FpCLEAs of LpNAL could be considered as potential biocatalysts for Neu5Ac synthesis. In addition, the biocatalyst could be reused without loss of activity for at least 10 cycles, which was higher than achieved with other described NALs.

2. Cloning and characterization of *Staphylococcus carnosus* TM300 NAL and its possible application to the production of neuraminic acid.

In order to complete the study carried out with LpNAL, another NAL from a non-pathogenic bacterium belonging to the group 1 NAL classification (Sanchez-Carrón., *et al* 2011), *Staphylococcus carnosus* NAL, was cloned and characterized. This NAL was selected from Uniprot (code B9DIJ2) showed significant identity with NALs from *Haemophilus influenzae, Clostridium perfringens, Lactobacillus plantarum* and *E. coli* K-12 (Uniprot code: P44539, Q9S4K9, P59407 and P0A6L4). This ScNAL was cloned and purified in two steps, showing the highest recovery (86%) yet described for NALs (Table 5.1). This recovery was 2- to- 4-fold higher than the recovery described for LpNAL and *Clostridium prefringes* NAL, whose purification was performed in three steps (42.3% recovery) and in 5 steps (21% recovery, respectively (Sánchez Carrón *et al*., 2011; DeVries & Binkley, 1972). The

kinetic and biochemical characterization revealed that their properties were very similar to those of other NALs studied, although it should be noted that the K_M for ManNAc and pyruvate were lowest described until now (Sánchez Carrón *et al*., 2011; Aisaka *et al*., 1991, Lee *et al*., 2008, DeVries & Binkley, 1972). This is important since the removing excess a pyruvate at the end of the reaction is an industrial problem. Thus, ScNAL was used for the synthesis of Neu5Ac, obtaining 94% conversion in 3 hours under optimized working conditions. This high conversion makes ScNAL a promising biocatalyst for the synthesis of Neu5Ac and its derivatives.

3. KDN production with immobilized enzyme recombinant FpCLEAs

After confirming that the NAL of *Staphylococcus carnosus* showed good biocatalytic characteristics for the synthesis of Neu5Ac, it was immobilized in the form of FpCLEAs to study its possible use as a biocatalyst for the synthesis KDN from D-mannose and pyruvate, since KDN is a derivative of sialic acid with interesting and different properties from those of Neu5Ac. The optimal conditions for the synthesis of KDN, were found to be a 1:2 D-mannose/pyruvate ratio, and a temperature of 37 ºC. Under these conditions, very high conversions (90%) compared with previously reported conversions (Salagnad *et al*., 1997; Wang *et al*., 2006) were possible in a short time (8 hours). This result reinforces the use of FpCLEAs as an interesting technique for obtaining valuable NAL biocatalysts and sialic acid derivatives, such as KDN.

4. Molecular characterization of N-acetyl neuraminate lyases from the previously unstudied group 3

In order to complete the study on NALs, and taking into account that there have been no previous studied of the NALs of Group 3, the cloning and characterization of two non-pathogenic members (*Lactobacillus sakei* and *Lactobacillus antri*) was carried out.

The study determined that its sequence showed 30% similarity with NALs of group 1 and 2. The experiments showed that group 3 NALs had a lower optimum pH and temperature than the NALs of group 1 and 2 (Li *et al.*, 2008; DeVries & Binkley, 1972, Sánchez-Carrón *et al.*, 2011; García-García *et al.*, 2012; Aisaka *et al.*, 1991). LaNAL was active towards D-arabinose, and was able to achieve 80% conversion into Neu5Ac derivate, called KDO, using LaNAL. To complete the study of group 3 NALs their kinetic parameters were determined for Neu5Ac hydrolysis and synthesis. The K_M for the hydrolysis were lower than those described for groups 1 and 2, whereas the K_M for synthesis using ManNAc was higher than for other NALs groups (Li *et al.*, 2008; DeVries & Binkley, 1972, Sánchez-Carrón *et al.*, 2011, García-García *et al.*, 2012; Aisaka *et al*., 1991). The catalytic efficiency (k_{cat}/K_M) for the hydrolysis of both representatives of groups 3 was similar to other previously reported NALs for these substrates (Sánchez-Carrón *et al.*, 2011; García-García *et al.*, 2012).

On the synthesis side, the k_{cat}/K_M for pyruvate was higher, especially in the case of LaNAL, compared with group 1 and group2 (Li *et al*., 2008, García-García *et al*., 2012).

To explain these different kinetic parameters of the NAL group studied, modeling was carried out and the active center was compared with crystallized NALs, the main change found being the substitution of Ser208 (EcNAL numbering) by a

Gly211 (LaNAL numbering). In addition, Val251 in *E. coli* also appears to be an important amino acid (Figure 7.9, b), and in group 3 NALs this residue is substituted by a bulky residue (Met) which hinders the binding of the substrate. Unfortunately, the mutant Met256Val was produced in the form of inclusion bodies. Furthermore, the presence of a proline (Pro193, LaNAL numbering), instead of a Tyrosine (Tyr 190, EcNAL numbering), could impose a forced conformation on the residues, Asp194 and Glu195 which participate in the binding of atoms O6, O8 and O9 of the substrate. In fact, the mutation Pro193Tyr caused a 3-fold increase in the K_M for ManNAc up to 915 mM (Table 7.3). To test the critical amino acids of the group 3 NAL, a double mutant was also constructed (Gly210Ser/Tyr213Gly), which led to a higher increase in the K_M for ManNAc up to 3.2 M.

To extend the above study between Group 3 NALs and other groups of NALs, an evolutionary analysis was performed to detect amino acid sites that show different degrees of evolutionary conservation among members of the NAL, using Diverge Detection Variability in Evolutionary Rates Software (Gu *et al*., 2002). This analysis showed that groups 3 and 1 were more divergent than groups 3 and 2. The divergent amino acids were basically located in the outer helices and a few were close to the β–barrel but did not affect the active site.

5. Molecular characterization of a new N-acetylneuraminate synthase from *Idiomarian loihiensis*

Finally, to complete the identification of potential biocatalysts for the synthesis of Neu5Ac, a N-acetylneuraminate synthase (NAS, EC 2.5.1.56), which catalyzes the condensation of N-acetylmannosamine (ManNAc) and phosphoenolpyruvate (PEP)

to form N-acetylneuraminic acid (sialic acid; Neu5Ac) in an irreversible reaction (Suryanti *et al*., 2003), of several possible candidates, the NAS gene from nonpathogenic *Idiomarina loihiensis* was studied.

The enzyme was active between pH 6.5 and 8.5, with maximum activity at pH 7 (Figure 8.3, a). This result was similar to that described for other NAS, such as those of *S. agalactiae* (Suryanti *et al*., 2003) and *E. coli* K1-M12 (Komaki *et al*., 1997). The enzyme showed a maximum temperature of about 40 ºC and was more thermostable than *S. agalitiae*. Its catalytic efficiency k_{cat}/K_M was 10-fold higher than that of *C. jejuni*, but 2-fold lower than that of *N. meningitidis*, indicating that IlNAS could be a promising biocatalyst. The combined study of its 3D modelling and the phylogenetic analysis pointed to ten conserved blocks in the NAS sequence that could be used to refines current databases and to ensure correct assignment of future sequences of bacterial NAS.

X. Conclusions

From the results present in this thesis, the following conclusion could be obtained:

1. N-acetyl neuraminate lyase from the non-pathogenic bacteria *Lactobacillus plantarum* was kinetically characterized in the synthetic reaction to produce sialic acid or Neu5Ac from N-acetyl mannosamine (ManNAc) and pyruvate, using both free enzyme and immobilized enzyme in the form of cross-linked enzyme aggregates (CLEAs) obtained by precipitation with 90% ammonium sulfate and crosslinking with 1% glutaraldehyde. This novel enzyme showed high catalytic activity and stability at alkaline pHs (pH>9) and a good temperature stability in the synthetic reaction, up to 60 ºC. These biotechnological relevant properties were enhanced by immobilization as new stabilized CLEAs in the presence of bovine serum albumin followed by a sodium borohydride treatment and final disruption by FastPrep (FastPrepCLEAs). This procedure showed a two-fold increase in activity of immobilized enzyme compared with other immobilization methods, and when used in the synthesis of Neu5Ac produced high conversion (77%) and good reusability.
2. In order to increase this conversion, a new N-acetyl neuraminate lyase from a GRAS microorganism *Staphylococcus carnosus* TM300 was cloned, overexpresed and purified with the highest expression (403 mg L^{-1} cultive) and recovery (86%) described for these type of enzymes. The recombinant enzyme showed similar kinetic parameters, optimum pH and temperature that the best described N-acetyl neuraminate lyase, making it promising and cheap biocatalyst. To confirm its biotechnological potential, Neu5Ac was synthesized in 3 hours under simple industrial working condition with a high degree of conversion (94%).

3. Due to the above characteristics of *Staphylococcus carnosus* N-acetylneuraminate lyase, it was also tested as biocatalyst in the production of Neu5Ac derivatives. To increase its stability, the enzyme was immobilized as a FastPrep CLEAs and the effects of substrate ratio and temperature were test to find optimal conditions. At pH 7.0 and 37 ºC, 90% conversion was achieved after 8 hours with a 2-fold excess pyruvate to D-mannose (1.2 M/0.6M, respectively). This conversion was high compared to the 20% previously described in the bibliography. This reinforces the use of FastPrepCLEAs of *S. carnosus* NAL as a possible industrial biocatalyst.
4. To open up more the range of N-acetylneuraminate lyase in the search of more efficient biocatalysts, a new phylogenetic N- acetyl neruaminate lyase group (group 3) was explored. Two candidates were also found among non-pathogenic bacteria, *Lactobacillus antri* and *Lactobacillus sakei*. After their cloning, expression and purification, these enzymes showed less activity, and low optimum pH and temperature compared to previously described NALs. Interestingly, these enzymes (LaNAL and LsNAL) showed higher K_M values than that of NALs from group 1 and 2 for ManNAc and lower for pyruvate. In order to explain these results, 3D structure of both enzymes were modeled finding that a replacement of a serine 208 (EcNAL, numbering) in the active center for a glycine 211 (LaNAL, numbering) reduced the number of hydrogen bonds formed with the substrate. In addition, substitution of valine 251 (EcNAL, numbering) for a bulky methionine 256 (LaNAL, numbering) in group 3 active center also reduced the activity towards the substrate. Morever, the presence of proline 193 (LaNAL, numbering) instead of a tyrosine 190 (EcNAL, numbering) also

forced conformation of the active center decreasing the binding of the substrate. To test *in silico* differences, some mutants were constructed, finding that the change Pro193Tyr produced a 3-fold increase in K_M for ManNAc.

5. Finally, a new N-acetyl neuraminate synthase from *Idiomarina loihiensis* was cloned, overexpressed and purified with high recovery (95%). The enzyme showed a 10-fold higher catalytic efficiency towards ManNAc than that of *Campylobacter jejuni* (0.2 vs 0.02 $mM^{-1}s^{-1}$), indicating that IlNAS could be a efficient biocatalyst at pH 7.0 and 37 ºC. Its structure was also modeled and phylogenetically compared with other putative sequences in the databases, finding the existence of ten conserved blocks, which could be used to refine current databases and to ensure correct assignment of future incoming sequences of bacterial NAS.

XI. Resumen en castellano

11.1 INTRODUCCIÓN

La industria química esta comprometida con el desarrollo sostenible para satisfacer las necesidades del presente sin comprometer las generaciones futuras. Es conocido, que los procesos biocatalíticos tienen varias ventajas en comparación con procesos químicos clásicos, ya que, debido a sus moderadas condiciones de reacción y su alta selectividad, la demanda de energía implicada y la cantidad de residuos producidos es relativamente baja. Sin embargo, a pesar de estas ventajas los procesos de biocatálisis, a menudo no son competitivos en comparación con los procesos clásicos, por lo tanto la biotecnología tiene aún que ponerse al día en lo que respecta a la mejora de los catalizadores e ingeniería de procesos. Un grupo de reacciones biocatalíticas interesantes son las condensaciones aldólicas entre un aldehído y una cetona o dos aldehídos catalizadas por aldolasas (aldehído-liasas, EC 4.1.2.x). Los productos, así obtenidos son de gran valor en el emergente campo de la glicobiotecnología (Faber 2004, Huang *et al*., 2007; Yu *et al*., 2006). Algunas de las enzimas más interesantes son las N-acetilneuraminato liasas (NAL; EC 4.1.3.3) y las N-acetilneuraminato sintasas (NAS; EC 2.5.1.56), porque catalizan la síntesis de ácido N-acetilneuramínico o ácido sialico (Neu5Ac), un amino azúcar a menudo presente en la posición terminal de células de mamífero. El ácido N-acetilneuramínico y sus derivados son compuestos interesantes con diversas aplicaciones terapéuticas.

11.1.1 Ácido siálico

El ácido siálico es un término genérico para designar una amplia familia de más de 50 azúcares estructuralmente distintos con un esqueleto de nueve átomos de carbono (Angata & Varki, 2002; Vimr *et al.*, 2004). Los ácidos siálicos más comunes

son, el ácido N-acetilneuramínico (Neu5Ac), que contienen el grupo N-acetilo en posición C5, el ácido 2-ceto-3-desoxi-D-glicero-D-galacto-nonulosonic (KDN) que contiene un grupo hidroxilo en la misma posición, el ácido 2-ceto-3-desoxi-D-mano-octulosonic (KDO) y el 3-desoxi-D-arabino-heptulosonate-7-fosfato (DAH-7-P). El grupo N-acetilo en sí mismo puede ser hidroxilado para producir ácido N-glicolilneuramínico (NeuNGc) (Figura 1.1). Más de 60 derivados de Neu5Ac y KDN son conocidos, y presentan alguna modificación en las posiciones C-4, C-7, C-8 y C-9 con acetato, lactato, sulfato, fosfato y metilo (Tao *et al*., 2010).

La historia del ácido siálico comienza a mediados de 1930, cuando el azúcar se aisló de mucina submaxilar por Gunnar Blix en 1936, dándole el nombre de "ácido siálico". Poco tiempo después, en 1940, Ernst Klenk identificó una sustancia que dio un color púrpura con el reactivo de Bial a la cual llamó ácido neuramínico, por ser constituyente de los glicolípidos derivados del cerebro. Más tarde se determinó que era el mismo compuesto aislado por Blix en la saliva y la nomenclatura se aclaró en 1957 (Blix *et al*., 1957), dando el nombre de "ácido siálico", que en la actualidad se refiere a una familia de más de 50 azúcares naturales (Angata & Varki, 2002), así como un número creciente de azúcares sintéticos (Keppler *et al*., 2001).

Neu5Ac y sus derivados tienen numerosas funciones fisiológicas importantes, que están estrechamente relacionados con la diversidad estructural de estas moléculas (Figura 1.2) (Schauer, 2000).

Las funciones principales se pueden dividir en las siguientes categorías:

1. Dotar de carga negativa a los glicoconjugados presentes en las membranas celulares.

2. Determinar la estructura macromolecular de ciertas glicoproteínas.
3. Transferencia de información entre células.
4. Reconocimiento específico de receptores y células

El ácido siálico se sabe que es una unidad de monosacárido clave en los gangliósidos cerebrales y glicoproteínas, que funcionan en el SNC para regular la migración de células, el crecimiento neurítico, el alargamiento de los axones, y la formación y la plasticidad sináptica (Kiss & Rougon, 1997; Rutishauser & Landmesser, 1996).

11.1.2 Interés médico y biotecnológico del ácido siálico

Neu5Ac es un intermediario para la síntesis de agentes antivirales, lo que ha dado lugar a una gran demanda de Neu5Ac en todo el mundo. Las sialidasas juegan un importante papel en la infección microbiana y pueden ser eficazmente inhibidas por derivados del Neu5Ac, por tanto estos pueden ser usados como fármacos para la prevención de dichas infecciones. Entre estos fármacos se encuentran actualmente el zanamivir (4-guanidino-Neu5Ac2) que presentan un fuerte efecto inhibidor sobre las sialidasas (Figura 1.3).

Por otro lado, el ácido siálico es una de las sustancias presentes en la leche materna considerada de vital importancia para desarrollar y mejorar las conexiones neuronales, que se deben establecer durante el periodo embrionario y los primeros años de vida. La sustitución de la lactancia materna, por leches infantiles con un bajo nivel de Neu5Ac puede llevar a un déficit nutricional, para evitarlo podría ser utilizado Neu5Ac como nutracéutico en dichas leches infantiles.

El principal inconveniente de su uso es el costo de la producción de Neu5Ac, que eleva su precio en el mercado a (50-100 $ g^{-1}). Este elevado precio es debido a su método convencional de preparación a partir de fuentes naturales como la leche, el huevo o el ácido colomínico. Se han planteado alternativas para abaratar este proceso, entre las que se encuentran la síntesis enzimática para la cual se necesita una fuente de la que pueda obtenerse las enzimas implicadas en su síntesis en cantidades industriales. Las enzimas bacterianas implicadas en la síntesis y degradación del Neu5Ac podrían ser una buena opción, pero para ello, es necesario previamente un estudio detallado de cómo funciona el metabolismo del ácido siálico entre las bacterias.

11.1.3 Metabolismo del ácido siálico en bacterias

11.1.3.1 Catabolismo del Neu5Ac

Debido a que los ácidos siálicos predominan en animales, sobre todo en mamíferos, es lógico pensar que existan microorganismos íntimamente asociados a ellos que sean capaces de realizar su metabolismo, bien sea como comensales o como patógenos. Diferentes estudios, en los que se han aislado mutantes de *E. coli* K1 incapaces de metabolizar el ácido siálico, han demostrado la presencia de un operón para el metabolismo del Neu5Ac inducible según su disponibilidad. Este operón cuenta con 4 genes: *nanT*, *nanA*, *nanE* y *nanK*. Los dos primeros fueron los inicialmente descubiertos, y su función está relacionada con el transporte del Neu5Ac al interior de la célula (*nanT*) y con la degración del mismo a *N*-acetil manosamina (ManNAc) y piruvato (*nanA*; que expresa la enzima NAL). Posteriormente se comprobó que *nanK* expresa una kinasa dependiente de ATP para ManNAc y que *nanE* expresa la epimerasa que genera GlcNAc-6P a partir de

ManNAc-6P. Desde entonces se ha considerado como sistema *nan* completo, aquel que mínimamente incluya estos cuatro genes.

La mayoría de los microorganismos fosforilan la ManNAc antes de su epimerización a *N*-acetil glucosamina (GlcNAc), como ocurre en *E. coli* (ver Figura 1.4). Sin embargo, ciertas bacterias como *Bacteroides fragilis*, difieren significativamente en este aspecto. Así en *E. coli* y otras baterias (casi todas patógenas), tras la liberación de ManNAc por NAL ésta es inmediatamente fosforilada por la enzima y epimerizada hasta GlcNAc, la cual entra a la ruta de degradación de azúcares (glicolisis). Por el contrario, el gen *nanE* de *B. fragilis* convierte ManNAc a GlcNAc sin necesidad de que esté fosforilado, actuando de una forma similar a como lo hace la proteína *N*-acetil-D-glucosamina 2-epimerasa (AGE) de mamíferos, también conocida como renin-binding protein (RnBP). La GlcNAc producida es entonces fosforilada por RokA kinasa y metabolizada hasta fructosa-6P para ser utilizada en la glicolisis. Estas enzimas descritas en la degradación del ácido siálico (AGE y NAL), también catalizan las reacciones reversibles, lo que hace posible su utilización en la síntesis de Neu5Ac.

11.1.3.2 El anabolismo de Neu5Ac

La biosíntesis de Neu5Ac sigue rutas similares en bacterias y mamíferos (Figura 1.5). El primer paso es catalizada por la enzima, UDP-N-acetil-glucosamina 2-epimerasa, que transforma UDP-N-acetilglucosamina (UDP-GlcNAc) en N-acetylmannosamine (ManNAc) (Tanner, 2005). En los mamíferos la UDP-GlcNAc 2-epimerasa fosforila ManNAc algo que no ocurre con la enzima de origen bacteriano.

En los mamíferos, Neu5Ac-9-fosfato sintasa cataliza la condensación de fosfoenolpiruvato (PEP) con ManNAc-6-P, generando Neu5Ac-9-P. La enzima bacteriana, es la sintasa de ácido siálico (Neu5Ac sintasa), que cataliza una condensación similar de ManNAc y PEP para generar Neu5Ac. Ambas rutas finalmente convergen para producir CMP-Neu5Ac. Este último compuesto es una forma activada de Neu5Ac, que es utilizado por CMP-sialil transferasas para transferir éste a oligosacáridos, polisacáridos o glicoconjugados. Neu5Ac sintasa se destaca como una diana terapéutica, ya que es exclusivo de la vía de bacterias y utiliza un sustrato diferente a su análogo en mamíferos (Tanner, 2005).

11.1.4 Enzimas implicadas en síntesis Neu5Ac

La enzima aldolasa o N-acetilneuraminato liasa (NAL, EC 4.1.3.3) es una aldolasa de clase I que cataliza la escisión del ácido N-acetilneuramínico en piruvato y N-acetil-D-manosamina a través de un intermedio de base de Schiff, con un equilibrio que favorece la hidrólisis del Neu5Ac. NAL también cataliza la reacción inversa de condensación aldólica y se ha utilizado de esta forma para sintetizar ácido siálico y algunos de sus derivados a partir de piruvato y N-acetil-D-manosamina (Figura 1.6) (Huang *et al*., 2007; Yu *et al*., 2004). La mayoría de NAL descritas hasta la fecha provienen de patógenos humanos: *Escherichia coli* (EcNAL) (Aisaka *et al.*, 1986; Ohta *et al*, 1986; Otha *et al*, 1985), *Clostridium perfringes* A99 (Traving *et al.*, 1997), *Haemophilus influenzae* (HiNAL) (Lilley *et al*., 1998), *Trichomonas vaginali*s (Meysick *et al*., 1996), *Pasteurella multocida* (Li *et al*., 2008). La estructura tridimensional del homotetrámero de NAL está formada por monómeros que presentan un barril $(\alpha/\beta)_8$, y tres α-hélices en el C-terminal. Su mecanismo de acción consiste en la formación de una base de Schiff en la que interviene un residuo lisina altamente

conservado. Esta lisina interactúa sobre el C2 del piruvato, que a su vez forma puentes de hidrógeno con residuos serina y treonina, también altamente conservados.

Otra enzima implicada en la síntesis de Neu5Ac es la N-acetilneuraminato sintasa (NAS; EC: 2.5.1.56) que cataliza la condensación irreversible de fosfoenolpiruvato (PEP), ya sea con la N-acetilmanosamina (bacterias) o N-acetilmanosamina-6-fosfato (mamíferos) para dar ácido N-acetilneuramínico o N-acetilneuramínico-6-fosfato, respectivamente (Figura1.10) (Tanner, 2005). Todas las NAS estudiadas hasta la fecha parecen ser metaloenzimas que requieren de un catión divalente para su actividad, y la mayoría muestran la mayor actividad en presencia de Mn^{+2} (Tanner, 2005). Hasta la fecha, sólo las estructuras de la sintasa de *Neisseria meningitidis* (PDB: 1xuu) han sido resueltas (Gunawan *et al.*, 2005.). La enzima se cristalizó en presencia de Mn^{2+}, PEP, y el sustrato no reactivo análogo N-acetilmanosaminitol (rManNAc o ManNAc reducida). Cada monómero de la enzima contiene dos dominios unidos por una región unión (linker) (Figura 1.12). El dominio N-terminal de mayor tamaño tiene el clásico plegamiento en barril TIM, con ocho hebras β-barril rodeada por ocho hélices. El dominio C-terminal que es más pequeño está compuesto por 65 residuos y es muy similar a los encontrados en las proteínas de anti-congelación tipo III del pescado (Jia *et al*., 1996). Dos de estos monómeros se disponen complementariamente N-ter de uno con la C-ter del otro para obtener la estructura homodimérica.

11.1.5 Inmovilización de Enzimas (CLEAs)

El desarrollo de una nueva familia de enzimas inmovilizadas en agregados de proteínas entrecruzados (CLEAS) es una alternativa económicamente eficiente a los procesos de inmovilización de enzimas. Estos se producen mediante precipitación en medio acuoso, por adición de sulfato amónico y entrecruzamiento con glutaraldehido. La metodología para la producción de CLEAs combina principalmente purificación e inmovilización en una operación única, ya que no requiere una enzima altamente pura (Figura 1.13). Las CLEAS son biocatalizadores muy atractivos, debido a su fácil preparación, precio y eficacia. Además las CLEAs pueden ser reutilizadas y muestran una mayor estabilidad y rendimiento. La metodología es esencialmente aplicable a cualquier enzima (Sheldon *et al.*, 2007).

11.2. OBJETIVOS

El objetivo principal de la presente Tesis Doctoral fue estudiar nuevas enzimas implicadas en la síntesis de ácido siálico o Neu5Ac. Por tanto, el estudio se centró en dos enzimas claves en el metabolismo de sínteis (N-acetil neuraminato sintasa) e hidrólisis (N-acetil neuraminato liasa) del ácido siálico, con el fin de encontrar nuevos biocatalizador procedentes de microorganismos no patógeno para la producción Nue5Ac y sus derivados. Este objetivo principal se puede dividir en los siguientes objetivos parciales.

1) Caracterización molecular e inmovilización de una N-acetil neuraminato liasa de *Lactobacillus plantarum* WFCS1 para la producción de Neu5Ac.

2) Caracterización molecular de una N-acetil neuraminato liasa de *Staphylococcus carnosus* para la obtención de Neu5Ac.

3) Inmovilización de N-acetil neuraminato liasa de *Staphylococcus carnosus* para la producción de un derivado de Neu5Ac, el ácido 2-ceto-3-desoxi-D-glicero-D-galacto-nonulosonic (KDN)

4) Caracterización molecular de N-acetil neuraminato liasas del grupo filigenético 3, previamente no estudiado, con el fin de encontrar posibles biocatalizadores para la síntesis de Neu5Ac.

5) Caracterización molecular de la N-acetil neuraminato sintasa procedente de un microorganismo no patógeno.

11. 3. MATERIALES Y MÉTODOS

11.3.1 DNA genómico

El DNA genómico usado para esta tesis fue obtenido de:

1. *Staphylococcus carnosus*. Dicho microorganismo fue utilizado para la obtención de una potencial N-acetilneuraminato liasa, el cual fue suministrado por la colección alemana de cultivos tipo (DSMZ). El microorganismo fue cultivado en medio YP.
2. *Lactobacillus plantarum.* . Dicho microorganismo fue utilizado para la obtención de una potencial N-acetilneuraminato liasa, el cual fue suministrado por la colección alemana de cultivos tipo (DSMZ). El microorganismo fue cultivado en medio YP.
3. *Lactobacillus sakei 23K*. Su DNA genomico fue generosamente donado por la Prof. (Monique Zagorec, Unité Flore Lactique et Environnement Carné, INRA, Domaine de Vilvert, F-78350 Jouy-en-Josas, France), y usado para la obtención de una potencial N-acetilneuraminato liasa

4. *Lactobacillus antri*. Su genómico fue directamente proporcionado por la colección alemana de cultivos tipo (DSMZ) (#16041), y usado para la obtención de una potencial N-acetilneuraminato liasa.

5. *Idiomarina loihiensis* L2TR. Su genómico fue directamente proporcionado por la colección alemana de cultivos tipo (DSMZ) (#15497), y usado para la obtención de una potencial N-acetilneuraminato sintasa.

11.3.2 Reactivos y cepas comerciales

Los sustratos para las enzimas se compraron a Carbosynth (Berkshire, UK), los cofactores, los inhibidores, los iones divalentes, EDTA, IPTG, antibióticos, los estándares de peso molecular y el bromuro de etidio se compraron a Sigma Aldrich (Madrid, España).

El sulfato amónico y las sales para los buffers fueron de Fluka. Los reactivos para la electroforesis y la cuantificación de proteínas (Bradford) fueron comprados a Biorad (Hercules, EEUU). Los disolventes orgánicos para el HPLC de Análisis Vínicos (Tomelloso, España) y el sistema de agua MilliQ de millipore (Bedford, EEUU). Los medios de cultivo se adquirieron a Pronadisa (Madrid, España). Los reactivos para biología molecular fueron comprados a NEB (Bervely, EEUU) a excepción de la *Pfu* polimerasa (Stratagene, Amsterdam, Holanda) y la T4 DNA ligasa (Roche, Mannheim, Alemania). Los oligos específicos para las PCR se pidieron a Isogen (Darmstad, Alemania), y las cepas de clonación y vectores de expresión usados fueron de Novagen (Tablas 3.1 y 3.2). Los kits de extracción y purificación de ácidos nucleicos fueron suministrados por Qiagen (Valencia, EEUU).

11.3.3 Herramientas bioinformáticas

Las secuencias de los genes fueron obtenidas de la página del National Center for Biotechnology Information (NCBI), y su alineamiento e identificación se llevó a cabo usando BLAST y ClustalW2. El modelo 3D de las proteínas se consiguió utilizando Geno3D y su visualización mediante PyMol.

En la realización de los arboles filogenéticos se usó MEGA 4.0 para la visualización y construcción de los árboles. Tres métodos diferentes fueron utilizados para la obtención de estos árboles: Bayesian análisis (BY), Maximum Likelihood (ML) y Neighbor Joining (NJ). Para el estudio de divergencia funcional se uso DIVERGE software (Gu & Vander, 2002).

11.3.4 Métodos microbiológicos

Como norma general, *E. coli* se cultivó a 37 ºC con agitación en medio LB. En caso de ser necesario el cultivo se suplementó con el antibiótico para la selección de las que contiene vectores. Para la producción de proteína en elevada cantidad fueron cultivados en TB con los antibióticos necesarios. La inducción del cultivo se realizó añadiendo IPTG a la concentración óptima e incubada a 30 ºC o a inferior temperatura.

E. coli se transformó por electroporación usando el método descrito por Dower *et al.,* (1988), utilizando un electroporador Biorad. Tras la recuperación en SOC durante 1 hora, las células se sembraron en placa de agar con antibiótico para la selección de las bacterias portadoras del vector.

11.3.5 Amplificación por PCR y clonación de fragmentos de DNA

Los métodos de clonación usados fueron básicamente los descritos por Sambrock *et al.,* (1989). Las condiciones estándares de PCR son las indicadas en la Tabla 3.3. Tras la PCR y la digestión con enzimas de restricción (del vector y el inserto), el producto fue clonado en un vector pET (pET28a), inducible por IPTG.

La mutagénesis dirigida se realizó siguiendo el método descrito por Ho *et al.,* (1989), utilizando *Pfu* Ultra II. Las condiciones de PCR y los plásmidos usados están indicados en las Tablas 3.4 y 3.5.

11.3.6 Purificación de proteínas

La producción de proteína se llevó a cabo en un fermentador de 5 L, con 4 L de medio TB, induciendo la expresión con IPTG a 30 ºC durante 12 horas. El cultivo obtenido se diafiltró en el buffer apropiado, y se concentró a través de una membrana de 500-kDa, previamente a su ruptura en un rompedor celular (MiniZetaII, Netzsch). El extracto crudo obtenido se trató con DNasa, y se concentró por ultrafiltración a través de una membrana de 50- ó 100-kDa (GE Lifesciences, Uppsala, Suecia). Después de ultracentrifugar a 40,000*g* la proteína fue purificada mediante cromatografía de afinidad, aprovechando la presencia de colas de histidina introducidas por los vectores pET en las enzimas. La enzima obtenida se desaló y se guardó con glicerol al 5 % y almacenadas a -20 ºC.

11.3.7 Metodología de proteínas

La cuantificación se realizó utilizando el método descrito por Bradford (1976). La resolución electroforética de proteínas se llevó a cabo realizando un SDS-PAGE, utilizando un gel concentrador al 4 % y un separador al 12 % de acrilamida:bisacrilamida. La electroforesis se realizó en un sitema Miniprotein III (Biorad, Hercules, EEUU), y las proteínas se tiñeron usando Azul Brillante de Coomasie (Sigma, Madrid, España). El marcador de peso molecular utilizado se compró a NEB (P7708S).

La determinación de la masa molecular de la enzima nativa se llevó a cabo mediante cromatografía por filtración en gel usando una columna Superdex 200 10/30 GL (GE Lifesciences). Este método además se utilizo para confirmar la homogeneidad de la proteína purificada. Para la determinación de la masa molecular bajo condiciones desnaturalizantes se utilizó la técnica HPLC acoplada a un espectrómetro de masas (HP/1100 LC/MSD Ion Trap System, Agilent technologies), la cual permite un cálculo exacto con una precisión de 1 Da.

Las curvas de fusión (melting) para la determinación de la desnaturalización de proteínas se realizó con la sonda SYPRO Orange (Molecular Probes, Paisley, Reino Unido). La desnaturalización se indujo incrementando la temperatura en un gradiente lineal de 20 a 100 ºC, utilizando un aparato de RT-PCR (modelo 7500, Applied Biosystems), y la gráfica del incremento de fluorescencia obtenida se utilizó para el cálculo de la T_m.

11.3.8 Ensayos espectrofotométricos

11.3.8.1 Ensayo enzimático para la N-acetilneuraminato liasa

La conversión de Neu5Ac en ManNAc y piruvato por NALs (ScNAL, LsNAL y LaNAL) se determinó utilizando como enzima acoplada lactato deshidrogenasa (EC: 1.1.1.27). Este método mide la disminución de la absorbancia a 340 nm que corresponde a la oxidación del NADH (ε_{340} = 6,200 M^{-1} cm^{-1}), producida por la lactato deshidrogenasa (LDH), debido al piruvato que apareció como consecuencia de la hidrólisis de Neu5Ac en dicho compuesto y ManNAc por NALs. El medio de reacción estándar contenía 150 µM de NADH, 0.5 U de LDH, 10 mM de Neu5Ac y NALs purificadas (4 µg ScNAL, 17 µg LsNAL y 1 µg LaNAL) en 20 mM de tampón fosfato sódico pH 7.

11.3.8.2 Ensayo enzimático para la N-acetilneuraminato sintasa de *Idiomarina loihiensis*

La conversión de ManNAc y PEP en Neu5Ac por IlNAS se determinó utilizando lactato deshidrogenasa (EC: 1.1.1.27) y N-acetilneuraminato liasa (ScNAL) como enzima acoplada. Este método mide la disminución de la absorbancia a 340 nm correspondientes a la oxidación del NADH producido por lactato deshidrogenasa (LDH) cuando el piruvato aparece como consecuencia de la hidrólisis de Nue5Ac por ScNAL, este Neu5Ac es sintetizado por IlNAS en presencia de ManNAc y PEP. El medio de reacción estándar contenía 150 mM de NADH, 0.5 U de LDH, 8 U ScNAL, 10 mM PEP, 10 mM de ManNAc, 1 mM $MnCl_2$ y 50 µg de IlNAS purificada en 20 mM de tampón MOPS pH 7.0.

11.3.9 Ensayos en HPLC

11.3.9.1 Ensayo enzimático para la N-acetilneuraminato liasa

La conversión de ManNAc y piruvato en Neu5Ac se determinó por HPLC-ELSD II (Shimadzu) utilizando una columna amino UK (Imtakt Co. Japón) y una fase móvil (58% de acetonitrilo: 42% acetato de amonio 50 mM) con un flujo de 0,4 ml / min y una temperatura de 60 º C. La reacción estándar contenía, 300 mM de ManNAc, 50 mM piruvato y 20 µg de purificada ScNAL en 20 mM de tampón fosfato sódico 7.0 pH. (El medio de reacción era muy similar para las otra NALs estudiadas)

11.3.9.2 Ensayo enzimático para la N-acetilneuraminato sintasa

La conversión de ManNAc y PEP en Neu5Ac se determinó por HPLC-ELSD II (Shimadzu) utilizando una columna amino UK (Imtakt Co. Japón) y una fase móvil (58% de acetonitrilo: 42% acetato de amonio 50 mM) funcionando a 0,4 mL/min a 60 º C. En estas condiciones, el tiempo de retención (RT) para Neu5Ac y ManNAc fueron 10.3 y 4.2 minutos, respectivamente. El medio de reacción estándar de contenia 10mM de ManNAc, 10 mM de PEP, 1 mM de $MnCl_2$ y 50 µg de IlNAS purificada en 20 mM tampón Tris-HCl pH 8.0.

11.3.10 Ensayos de estabilidad

El efecto de la temperatura y el pH se ensayó incubando la enzima durante periodos de tiempo determinados a diferentes temperaturas y pHs, y posteriormente, midiendo en condiciones estándar. De la misma forma se comprobó el efecto de cationes divalentes y agentes quelantes.

11.4 RESULTADOS Y DISCUSIÓN

Capítulo IV: Caracterización molecular de la N-acetilneuraminato liasa de *Lactobacillus plantarum* WCFS1 y su inmovilización como FastPrep CLEAs para obtener Neu5Ac.

La enzima Neu5Ac aldolasa (EC 4.1.3.3), también conocida como N-acetilneuraminato liasa (NAL) o ácido siálico aldolasa, cataliza la hidrólisis de ácido N-acetilneuramínico (ácido siálico, Neu5Ac) hasta piruvato y N-acetil-D-manosamina (ManNAc). Esta realiza también la reacción inversa de condensación aldólica para la síntesis de ácido N-acetilneuramínico (Neu5Ac) y algunos de sus derivados, a partir de piruvato y N-acetil-D-manosamina (ManNAc) (Shauer *et al*., 1982). Neu5Ac participa en diversas funciones biológicas, en las que actúa como receptor para microorganismos, virus, toxinas y hormonas. Esto la convierte en una molécula de interés para la producción de nuevos compuestos con aplicación terapéutica: inhibidores de la neuraminidasa (Zinamavir ó "Relenza®", GlaxoSminthKline) que actúan como antivirales efectivos contra la gripe aviar o aviárica (Liese *et al*., 2006; Itzsein, 2007).

El microorganismo *Lactobacillus plantarum*, del cual se ha clonado el gen para la obtención de la enzima, es una bacteria del ácido láctico, comensal del tracto digestivo humano y que ha sido usada como suplemento probiótico teniendo la consideración de de microorganismo GRAS. Esto hace que *L. planatrum* pueda ser considerada como una fuente de nuevas enzimas recombinantes en procesos de producción industrial, como es el caso de su N-acetil neuraminato liasa (LpNAL). Desde la segunda mitad del siglo pasado, numerosos esfuerzos se dedicaron al desarrollo de diversas enzimas inmovilizadas con el objetivo de facilitar su uso en

procesos continuos, ya que pueden reducir los costos al permitir la separación eficiente, el reciclaje y la reutilización de las mismas. Entre los diferentes modelos de inmovilización, destacan el procedimiento de fabricación de CLEAs (Cross-linked Enzyme Aggregates), ya que no necesitan soporte inerte adicional (Cao et al., 2000). Este método consiste en la precipitación de la enzima y el entrecruzamiento de la proteína usando reactivos bi-funcionales. El entrecruzamiento previene la pérdida y la posible solubilización de los agregados después de eliminar el agente de precipitación. Algunos aditivos se han propuesto para la estabilización de CLEAS como la albúmina de suero bovino (BSA) y borohidruro de sodio (SB)(Shah *et al*., 2004; Wilson *et al*., 2004). Para aumentar su reutilización se realizaron estudios de los efectos estabilizadores de las sustancias descritas anteriormente (SB y BSA) actuando conjuntamente, así como el uso de un dispositivo mecánico de dispersión rápida (FastPrep), lo que permite obtener CLEAs de tamaño menor que facilita la difusión de sustrato y una mayor homogeneidad de tamaño lo que mejora la estandarización del proceso(Montoro-García *et al*., 2010).

En este capítulo de la Tesis se ha llevado a cabo la optimización de la producción de FpCLEAs con LpNAL, además se estudio el caso de FpCLEAs para la síntesis de Neu5Ac.

En la producción de CLEAs se han utilizado diversos aditivos, como la albumina de suero bovino (BSA) y el borohidruro sódico (SB) para mejorar sus propiedades y reutilización, se ha observado que la co-precipitación de LpNAL con BSA, así como su posterior tratamiento con SB favorecía la obtención de CLEAs con una mayor

poder catalítico, llegando a presentar hasta un 82% de actividad sobre la nativa y una reutilización de más de 10 ciclos sin pérdida aparente de actividad.

Se han observado propiedades bioquímicas interesantes, ya que LpNAL muestra una estabilidad al pH básico no vista hasta el momento para otras NALs, siendo estable a pH 9 durante 15 días.

Los parámetros cinéticos para la actividad sintética se han investigado tanto con enzima libre como con las mejores FpCLEAs usando como sustrato ManNAc y piruvato. En ambos casos, FpCLEAs y enzima libre, y para ambos sustratos se ha observado una cinética de tipo Michaelis-Menten. La K_M aparente para la enzima inmovilizada para ManNAc (245 mM) fue 1.5 veces mayor que para la enzima libre(160 mM), mientras que para el piruvato, la K_M de la enzima inmovilizada (9 mM) era 2.2 veces menor que en el caso de la enzima libre (19.9 mM). Estos datos indican que la interacción de la enzima y el sustrato es más fuerte en el caso de piruvato, y no en el de ManNAc, indicando que tras la reticulación con glutaraldehído, se han podido generar obstáculos moleculares para este último sustrato. También es este el responsable de la disminución de la eficiencia catalítica (k_{cat}/K_M) de la enzima para ambos sustratos.

Finalmente se ha sido estudiado el potencial que presentan dichas CLEAs para la conversión de ManNAc a Neu5Ac, y se ha obtenido un rendimiento del 77% tras 15 horas.

Capítulo V. Caracterización de una nueva N-acetylneuraminato liasa de *Staphylococcus carnosus* TM300 y su aplicación para la producción de ácido N-acetilneuramínico.

Ácido N-acetilneuramínico (Neu5Ac) es una unidad de monosacáridos claves en los gangliósidos cerebrales y glicoproteínas, incluyendo el ácido poliN-acetilneuramínico de moléculas de adhesión celular neural (NCAM), cuya función en el Sistema Nervioso Central tiene por objeto regular la migración celular, el crecimiento de las neuritas, la elongación axonal, la formación sináptica y la plasticidad (Kiss & Rougon, 1997; Rutishauser & Landmesser, 1996). Concentraciones de Neu5Ac en tejidos corticales del cerebro se correlacionan con el desarrollo evolutivo en los animales superiores, y cabe resaltar que la concentración de Neu5Ac en la corteza frontal de los bebes alimentados con lactancia materna es mayor que en lactantes alimentados con leches preparadas (Wang *et al*., 2003). En conjunto, estos hallazgos sugieren la posibilidad de suplementar las leches preparadas para lactantes con Neu5Ac exógeno, ya que el Neu5Ac se encuentra en altas concentraciones en leche humana y no en fórmulas preparadas a partir de leche de vaca o de hidrolizados de proteínas. Sin embargo, uno de los principales obstáculos para el uso de Neu5Ac en alimentos enriquecidos es su elevado precio. Esto ha restringido su uso exclusivamente en la industria farmacéutica para la preparación de intermedios avanzados de antivirales, como el Relenza ® (GSK) (Liese *et al*., 2006; Itzstein, 2007; García-García *et al*., 2011).

La enzima N-acetilneuraminato liasa (NAL, EC 4.1.3.3) cataliza la hidrolisis del ácido N-acetilneuramínico en piruvato y N-acetil-D-manosamina y también la reacción inversa de condensación aldólica por lo que ha sido usada para sintetizar

ácido N-acetilneuramínico y algunos de sus derivados. Como el objetivo es hacer un enzima de aplicación en la industria alimentaria se ha buscado un microorganismo GRAS, en este caso *Staphylococcus carnosus* como fuente nuevas enzimas.

Mediante técnicas de biología molecular, y a partir del genómico de *S. carnosus* TM300, se ha clonado el gen de la NAL en un vector de expresión (pET28a). La proteína codificada por dicho gen ha sido sobreexpresada en un fermentador de 5 L y purificada mediante un proceso en dos pasos que incluye ultrafiltración tangencial y cromatografía de afinidad en una columna HisTrap mediante FPLC. El rendimiento de la enzima obtenida, electroforéticamente pura, es de un 86%.

La enzima así purificada presenta un pH óptimo de 7.0 y una temperatura óptima de 50 ºC para la actividad sintasa. Para la actividad hidrolasa presenta el mismo pH óptimo pero la temperatura óptima de reacción es mayor (60-70 ºC). También se ha estudiado la termoestabilidad de la ScNAL en su actividad sintética, observándose que la proteína mantiene el 60% de su actividad tras 2 horas a 60ºC, además se ha estudiado la estabilidad a pH alcalinos y se ha observado que mantiene el 80% de su actividad a pH 8 tras más de 4 horas de incubación a 37 ºC a ese pH. Para completar el estudio de la ScNAL se ha estudiado el efecto de diferentes tampones, así como la presencia de aditivos sobre su T*m*, mostrando que tanto la hidroxiectoina como el sulfato amónico mejoran la estabilidad de la enzima. Además se ha demostrado que la presencia de piruvato es la que más aumenta al estabilidad de la enzima alcanzando una T*m* máxima de 78 ºC e a 80 mM de piruvato.

Dicha enzima ha sido caracterizada cinéticamente, obteniéndose los valores de K_M y K_{cat} /K_M para la actividad hidrolasa sobre Neu5Ac de 2 mM y 2 $mM^{-1}s^{-1}$ respectivamente, dichos parámetros para la actividad sintetasa han sido, 149 mM y 0.03 $mM^{-1}s^{-1}$ para N-acetilmanosamina y de 14 mM y 0.21 $mM^{-1}s^{-1}$ para piruvato, respectivamente.

Finalmente se ha investigado el potencial de ScNAL para la producción de Neu5Ac, por condensación de ManNAc y piruvato, obteniéndose un porcentaje de conversión del 94 % en 4 horas usando 12 U/mL de ScNAL. Por lo tanto ScNAL puede ser posible biocatalizador para la síntesis de Neu5Ac para uso alimentario y farmacéutico.

Capítulo VI: Sin publicar

Capítulo VII: Sin publicar

Capítulo VIII: sin publicar

CONCLUSIONES

A partir de los resultados presentes en esta tesis, fueron obtenidas las siguientes conclusiones:

1. N-acetil neuraminato liasa de una bacteria no patógena *Lactobacillus plantarum,* fue caracterizada cinéticamente en la reacción de síntesis para producir ácido siálico a partir de N-acetil manosamina y piruvato, utilizando tanto la enzima libre como la enzima inmovilizada en forma de agregados enzimáticos (CLEAs), obtenidos por precipitación con 90% de sulfato de amonio y posterior reticulación con glutaraldehído al 1%. Esta nueva enzima mostraba una alta actividad catalítica y gran estabilidad a pHs alcalinos (pH> 9) así como una buena estabilidad a la

temperatura en la reacción de síntesis, llegando hasta 60 º C. Estas propiedades biotecnológicas han mejorado tras la inmovilización en CLEAs con estabilizadores como albúmina de suero bovino y borohidruro sódico y un nuevo método para la disrupción final, como es el FastPrep, a estas nuevas CLEAs las hemos denominado FastPrepCLEAs. Este procedimiento dio lugar a un aumento del doble de actividad en la enzima inmovilizada, en comparación con otros métodos de inmovilización, obteniéndose una conversión de 77% para la síntesis de Neu5Ac y una buena capacidad de reutilización.

2. Con el fin de aumentar la conversión en la síntesis de ácido siálico, una nueva N-acetil neuraminato liasa de un microorganismo GRAS, *Staphylococcus carnosus* TM300 se clonó, sobreexpreso y purificó obteniéndose la expresión y recuperación más alta descrita para este tipo de enzimas. La enzima recombinante mostraba similares parámetros cinéticos, pH y temperatura óptimos que los descritos para otras N-acetil neuraminato liasas, por lo que esta enzima se presenta como un prometedor biocatalizador. Para confirmar su potencial biotecnológico, Neu5Ac fue sintetizado en 3 horas bajo condiciones industriales de trabajo, con un alto grado de conversión (94%).

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