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ALAIN EDUARD MONSALVE MERA

Structural and enzymatic features of a recombinant β-fructofuranosidase from *Bifidobacterium adolescentis*

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With gratitude,

to mom, for walking the thousands extra miles for us.

With love,

to Yuly, for giving me the precious fruit of her being.

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“We are all in the gutter, but some of us are looking to the stars”

Oscar Wilde

ABSTRACT

MONSALVE ALAIN, M. **Structural and enzymatic features of a recombinant β-fructofuranosidase from *Bifidobacterium adolescentis*.** 2016. 92 p. Dissertation (Master in Science) - Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, 2016.

Despite the fact that Glycosyl Hydrolase Family 32 present 4 467 enzyme entries, only 14 of them have been characterized structurally. From the ten protein crystal structures deposited for *Bifidobacterium adolescentis* ATCC 15703 at PDB just one enzyme is related to the processing of non-digestible sugars and there is no structure of a β-fructofuranosidase. In this research we studied the biochemical properties and the structural features of a recombinant β-fructofuranosidase (BaFFse) from the healthy gut bacteria *B. adolescentis* ATCC 15703 (gen BAD_1325) heterologously expressed in *Escherichia coli* Rosetta. The enzyme was purified by nickel ion affinity chromatography and molecular exclusion chromatography; the purification process was judged by denaturing SDS-PAGE gel. Sucrose was used as a substrate for the enzyme activity assays and the amount of reducing sugars, detected by Dinitrosalycilic acid, was taken as indicator of the optimum conditions of hydrolysis for the enzyme. BaFFase crystal, grown in PEG 8K 25% (w/v) and buffer MES 0.1M pH 6.5, was diffracted at 2.44 Å and processed using the CCP4 program package. The enzyme presented a classical four-stranded five-bladed β-propeller and a C-terminal β-sandwich characteristic from the GH 32 family; however, connected to the β-propeller through a loop of 38 residues, BaFFase also presented an N-terminal β-sandwich domain, which sequence (residues 3-100 from BaFFase) did not match with any protein sequence when aligned against PDB database. Assays with Gel filtration calibration, DLS and SAXS showed that the enzyme was a stable homodimer in solution. Based on the superposition of structures using the a β-fructofuranosidase from *B. longum* KN29.1 we could deduced the three key aminoacids involved in the transferring of fructosyl moieties by BaFFase. A nucleophile attack is performed by the carboxylate of Asp 131, forming the fructose –BaFFase intermediate; Glu 375 donates a proton, acting as an acid base catalyst and Asp 269 stabilizes the transitions state in the fructosyl transferring activity. This is the first GH32 oligomeric enzyme belonging to the bacteria kingdom. We have described a novel additional β-sandwich domain for a GH32 enzyme that increases the region of contact to form a dimer. This is the first β-fructofuranosidase crystal structure from the microorganism *B. adolescentis* ATCC 15703.

Keywords: β-fructofuranosidase. GH32. Crystal structure. *Bifidobacterium adolescentis.*

RESUMO

MONSALVE ALAIN, M. **Aspectos estruturais e funcionais de uma β-fructofuranosidase recombinante da *Bifidobacterium adolescentis*.** 2016. 92 p. Dissertação (Mestrado em Ciências) - Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, 2016.

O presente trabalho disserta sobre os estudos das propriedades bioquímicas e as características estruturais de uma β-frutofuranosidase recombinante (BaFFse) da bactéria *Bifidobacterium adolescentis* ATCC 15703 (gen BAD_1325) presente em intestinos saudáveis. A proteína foi expressa heterologamente em *Escherichia coli Rosetta*. A enzima foi purificada por cromatografia de afinidade (íons de níquel) e cromatografia de exclusão por massa molecular; o processo de purificação foi avaliado por gel desnaturante tipo SDS-PAGE. A sacarose foi usada como substrato para os ensaios de atividade enzimática e a quantidade de açúcares redutores, detectados por ácido dinitrosalicílico, foi tomada como indicador das condições ótimas de hidrólise para a enzima. Cristais de BaFFase, crescidos em solução contendo PEG 8 k em tampão Hepes pH 6,5, foram difratados a uma resolução de 2,44 Å e processados utilizando o pacote de programas CCP4. A enzima apresentou um clássico enovelamento tipo composto por cinco pás de quatro-fitas β cada e um domínio C-terminal sanduíche-β característico da família GH32; no entanto, ligado ao *β-propeller*, através de um loop de 38 resíduos, a BaFFase apresentou um inédito domínio N-terminal sanduíche-β (resíduos 3-100 de BaFFase) ainda sem precedentes, quando alinhado contra a base de dados PDB. Ensaios de gel filtração, DLS e SAXS mostraram que a enzima se apresenta como um homodímero estável em solução. Com base na superposição estrutural, utilizando uma β-frutofuranosidase de *B. longum* KN29.1, foi possível inferir os três aminoácidos essenciais envolvidos na transferência de unidades de frutosil pela BaFFase. Um ataque nucleofílico é realizado pelo grupo carboxílico do Asp131, formando um intermediário frutose-BaFFase; o Glu375 doa um próton, atuando como um catalisador ácido-base e o Asp269 estabiliza o estado transições na atividade de transferência frutose. Um novo domínio sanduíche-β adicional para uma enzima GH32 é descrito. Esse domínio é responsável pelo aumento da região de contato e essencial para a formação do homodímero. Esta é a primeira estrutura cristalina da β-frutofuranosidase do microrganismo *B. adolescentis* ATCC 15703, além de ser a primeira enzima GH32 descrita neste estado oligomérico pertencente ao reino das bactérias.

Palavras-chave: β-frutofuranosidase. GH32. Estrutura cristalográfica. *Bifidobacterium adolescentis.*

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LIST OF ABREVIATIONS AND ACRONYMS

BaFFase	*B. adolescentis* ATCC 15703 β-fructofuranosidase
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
CAZy	Carbohydrate-active Enzymes
DLS	Dynamic Light Scattering
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
FFase	Fructofuranosidase
FRU	Fructose
FTase	Fructosyltransferase
GH32	Glycosyl Hydrolase family 32
GH68	Glycosyl Hydrolase family 68
LIC	Ligation-Independent Cloning
MES	2-(N-morpholino)ethanesulfonic acid
PDB	Protein Data Bank
PMSF	Phenylmethylsulfonyl fluoride
ORF	Open Reading Framework

CONTENT

1 INTRODUCTION

1.1 Saccharose

Saccharose is an obsolete name for sucrose, which is a disaccharide, composed of the combination of glucose and fructose: α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside or β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside. The term sucrose was coined by William Miller, form the French *sucre* and the chemical suffix for sugars – *ose*.[1]

Sucrose is the major disaccharide in the carbohydrate translocation and storage in plants,[2] and in nature, sucrose is present to store energy primarily from photosynthesis.[3] Sucrose is biosynthesized from UDP-glucose and fructose 6-phosphate by the enzyme sucrose-6-phosphate synthase using the energy gained by cleavage of Uridine diphosphate (UDP), and chemical synthesis of sucrose was first achieved by Raymond Lemieux in 1953.[4]

Its chemical formula is $C_{12}H_{22}O_{11}$ that corresponds to a molar mass of 342.30 g/mol, with an white appearance, a density of 1.587 g/cm^3 (solid) and a solubility in water of 2 100 g/L (25 °C). Sucrose crystallizes in the monoclinic space group $P2_1$ with parameters $a = a =$ 1.08631 nm, b = 0.87044 nm, c = 0.77624 nm, β = 102.938°.[5-6]

For the industrial production of sucrose two important sugar crops predominates: sugarcane (*Saccharum spp.*) and sugar beets (*Beta vulgaris*) in which sugar can account for 12% to 20% of the dried weight. The sucrose is extracted from these crops with hot water; concentration of the extract gives syrup form which solid sucrose can be crystallized. The liberated sugars can also be used in fermentation processes, and then transformed to ethanol, which with later distillation process could be used as a fuel. Alternatively, the energy got in the glucose-fructose linkage of sucrose can be used as energy to form polymers of fructose (Fructooligosaccharides) with the help of enzymes such as fructosyltransferases or fructofuranosidases- these terms will be explained in section 1.4.4. Those fructose polymers are prebiotic components with well-known health benefits.

1.2 Importance of enzymes to the feedstock

The industrial enzyme market comprises various enzymes types such as carbohydrases, proteases, non-starch polysaccharides, and others which includes phytases, lipases, catalases, and tannases. Those enzymes are spread over various application within food and beverage, cleaning agents, animal feed, and others.[7]

The industrial market of enzymes is estimated to grow at 7.0% from 2015 to 2020 passing from USD 4.2 billion (2014) to 6.2 billion at 2020.[7] From the above enzymes, carbohydrases are the most relevant as it has been reported to grow to 7.5% from 2015 to 2020.[7]

The food and beverage segment will dominate the industrial enzyme market, reaching USD 2.0 Billion for 2020.[7] Therefore, it is economically important to research about enzymes related with the food industry in general and with the production of additive for the beverages in particular, like the production of FOS, a prebiotic additive widely used in lactic beverages that can be produced through transfructosylation by beta-fructofuranosidases, fructosyltransferases, inulinases, among others. In this research, several studies with the beta-fructofuranosidase from the bacteria *Bifidobacterium adolescentis* ATCC 15703 will be presented.

1.3 Fructooligosaccharides (FOS)

Fructooligosaccharides are polymers of fructose linked to a glucose found in plants and can also be naturally produced by specially bacteria and yeast by with the help of enzymes that have the capacity to transfer fructose to another sugar (fructosyltransfering activity). In this section, we would like to explain what definition is going to be used in our research, as the name “fructooligosaccharides” seems to present various definitions.

Even that the term FOS by itself is ambiguous, since the number of fructoses varies,[8] it is accepted to call simple fructose oligomers as “FOS”, which are mainly composed of 1-kestose (GF2), nystose (GF3), and 1F-fructofuranosyl nystose (GF4) in which fructosyl units (F) are bound at the β-2, 1 position of sucrose (GF), respectively.[9] Some researchers agree that one can call FOS n if the polymerized chain varies from 1 to 12 units of fructose, while longer chains are considered as fructan polymers such as inulin-type, inulin neo-series, levan and mixed type of fructans. However, the IUPAC shows that the dividing point between oligo

and poly-fructosaccharides is 10.[8] Therefore, in this research we are going to name as a fructooligosaccharides to polymers with less than 10 units of fructose.

The most common types of fructooligosaccharides are 1-kestose (GF_2), Nystose (GF_3) and fructosyl nystose (GF_4), as shown in figure 1.

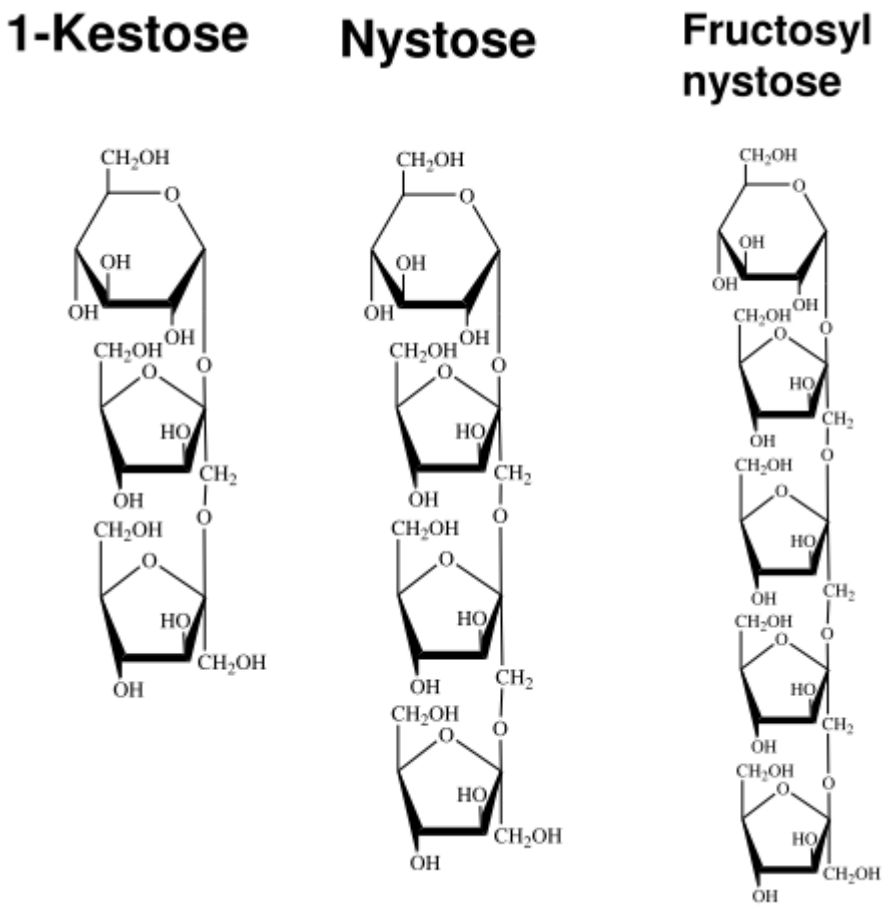

Figure 1 – Principal short chain fructooligosaccharides: 1-kestose, Nystose and Fructofuranosylnystose

Source: Adapted from OMORI et al.[10]

1.3.1 Health benefits of FOS

The term functional food is used to describe nutrients that have an effect on physiologic process that is apart from their nutritional function, and some of these nutrients are propounded to promote gastrointestinal mucosal integrity.[11] Once FOS are non-digestible sugars, they are not degraded or adsorbed at the stomach or in the small intestine and reach the colon intact. At the colon, they promote the proliferation of Bifidobacteria, which constitutes a great part of the intestinal micro flora[12] FOS have got very well demonstrated prebiotic properties[11–17] and can be considered as the most commonly used prebiotic fibers in

the production of functional foods formulations[12] because they are regarded as safe (GRAS) from the Food Drug and Administration, USA, and worth about 150 euros per kilogram.

The synthetic process of FOS was first developed in Japan in 1980 by Meija Seika Kaisha Limited under the trade name Meiologo.[18] Later on, other countries started to sell FOS under names like Actilight (Beghin-Meiji Industries France) or Nutraflora (USA). The Infant Formulas (IFs) are supplemented with prebiotics to exert similar effects to those of the breast milk; it is usually used from 1.6 to 5.0 g of FOS for each 100 g of Infant formulas [13]. FOS are also used as sweetener for diabetics because of its low calorimetric value, low glycemic index, moderate sweetness, and non-digestibility by humans; moreover, it have been observed to reduce the carcinogenesis.[19]

The use of prebiotics to orchestrate the gut microbiota composition is an emerging topic of the utmost biotechnological interest.[20]

1.4 Gut microbiota

Microbiota is a microbial community [21] and gut microbiota is the bacteria community located in the gut such as Bacterioides, Bifidobacterium, and Firmicutes. In the colon, specifically Bacteroides spp., bifidobacteria, enterococci, clostridia, and a few other groups dominate it.[22]

Microbiota of mammalian intestine depends largely on dietary polysaccharides as energy sources.[23] In the case of herbivorous, their diet is almost completely of cell-wall plant polysaccharides that are not digestible by host enzymes, and 70% of the total gut volume is devoted to microbial fermentation.[24] In omnivorous mammals, notably humans, the contribution of large-intestinal microbiota is approximately 10%.[25]

The breakdown of cellulose and other complex carbohydrates by microbiota from the gastrointestinal tract leads to the production of VFA, also known as short-chain fatty acids. The main VFA are acetate, propionate and butyrate in a ratio typically varying from 75:15:10 to 40:40:20.[25]

Most of the butyrate is converted to ketone bodies of CO2 by the epithelial cells. Propionate is largely converted to glucose and acetate is used principally by peripheral tissues, especially fat and muscle.[25]

Eventually, the acetic and the lactic acid produced by bacteria of the colon, e.g. *B. adolescentis*, can be converted to butyric acid by Firmicute bacteria such as Eubacterium sp and Anaerustipes sp,[26] then in this case *B. adolescentis* also contribute with the energy balance of their host.

The microbial community established at the human being exerts a profound effect on human health and physiology, providing benefits such as immune development, digestion of recalcitrant dietary nutrients [23] and inhibition of pathogens colonization.

In addition to the energetic contributions of VFA, produced from fermentation by the GIT microbiota, the VFA may indirectly influence cholesterol synthesis, help regulate insulin and glucagon secretions, have an effect on epithelial cell growth, blood flow and the normal secretory and absorptive functions of the GIT.[25]

Supplementation with galacto-oligosaccharides or inulin was shown to increase the abundance of Bifidobacteria in average, but certain volunteers were found to be non-responders.

1.4.1 Bifidobacterium genus

Bifidobacterium was first isolated from feces of breast-fed infants by Tissier at Pasteur Institute. An Italian scientist also discovered a bacteria in similar conditions as described by Tissier and both agreed to name this two organism as *Lactobacillus bifidus*.[27] Even that the classification of this microorganism was incorrect, it represents the first description of a bacteria from the Bifidobacteria genus. The initial descriptions used the word bifidus, based on the morphology, as the bacteria usually exist in the “Y”-shape or bifid form[27] (Fig. 2).

Bifidobacterium is a genus of gram-positive, nonmotile, non-spore-forming, and branched[27] obligate anaerobe bacteria that belongs to the Actinomycetales branch of the high – G +C Gram-positive that also include the corynobacteria, mycobacteria, and Streptomyces.[28] Bifidobacterium are ubiquitous inhabitants of gastrointestinal tract, vagina[27] and mouth of mammals, including humans.

A rapid identification of Bifidobacterium, and differentiation from Lactobacilli, could be done by analyzing the presence of 6-phosphate-phosphoketolase (F6PPK; EC 4.1.2.22), and the lack of aldolase and glucose-6-phosphate dehydrogenase, which are present in

Lactobacilli.[27] However, with recent techniques, such as DNA probes and pulsed-field electrophoresis, it is possible to differentiate more accurately Bifidobacterium strains from Lactobacillus, by evaluating the percentage of guanine and cytosine (G + C) in the corresponding DNA.[27] Lactobacillus contains <50 mol % G + C in DNA, whereas all members of the genus Bifidobacterium contain >50 mol % G + C.[27]

All lactic acid producers have been allocated within two families: Clostridium and Actinomycetes. The Actinomycetaceae family consist of five genera: *Bifidobacterium*, *Propionibacterium*, *Microbacterium*, *Corynebacterium*, and *Brevibacterium*.[27]

From the thirty-two species that have been found for *Bifidobacterium* genera (G+C bases content between 55-66%), 14 are present in humans, 12 in animals, and 3 in honeybees. A few have been isolated from human vagina and oral cavity, but the vast majority are from the gastrointestinal tract (GIT).[27]

Bifidobacterium adolescentis (figure 2) presents 58.9 % G+C bases, and habits the vagina and feces of humans, bovine rumen and sewage.[27]

B. adolescentis ATCC 15703 complete genome was registered at NCBI (National Center for Biotechnology Information, NIH, USA, Bethesda) under the taxonomy ID: 367928 on December 2006 by Lab Science Research Center from Gifu University, Japan. The microorganism was described as a normal bacteria inhabitant of the healthy human gut. *B. adolescentis* ATCC 15703 present 1631 protein sequences and only ten protein crystal structures have been described for this bacteria according to NCBI[29] and PDB,[30] consulted on July 2016. Most of the protein structures are enzymes synthases (glutamine synthase, polyketide synthase, etc) and only one enzyme structure is related to the processing of non-digestable sugars (PDB ID: 318B, the crystal structure of xylulose kinase).

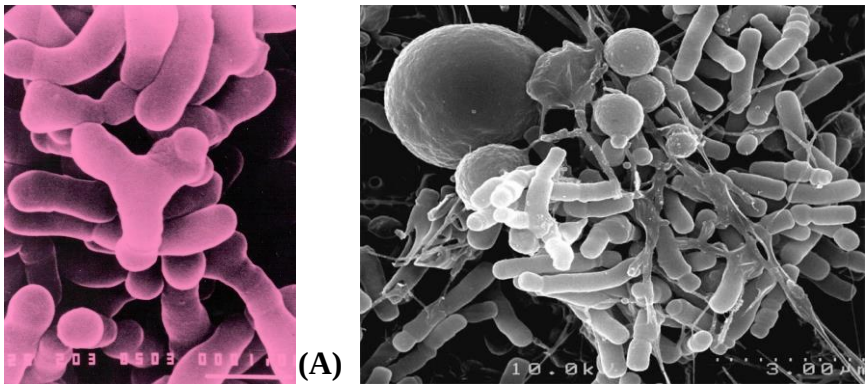

Figure 2 - (A) *Bifidobacerium spp*. (B) *B. adolescentis*.

Source: Adapted from JOANA [99]; WILLIAMS [100]

1.4.2 Importance of Bifidobacteria genus in health

Bifidobacterium has been found especially in the large intestine, which is characterized by neutral to mildly acidic pH, and presents by far the largest microbial community, dominated by obligate anaerobes[20] like *B. adolescentis*.[28]

The intestinal microbiota exerts a barrier against pathogenic bacteria in the digestive tract. [31] In particular, the gender *Bifidobacterium* has also been recognized with many of these benefits especially for the gastrointestinal health of new-born.

The gastrointestinal tract of a normal fetus is sterile.[32] Bifidobacteria are one of the first colonizers of the sterile gastrointestinal track (GIT) of new-born and predominates still breast-fed infant weaning, when they are surpassed and the diversity of the microbiota increases.[32]

During birth process, bacteria from the mother and surrounding colonize the gastrointestinal track. The microbiota of a breast-fed infant is rapidly dominated by Bifidobacterium and may repress the presence of other obligate and facultative anaerobes, and a more diverse microbiota develops only after dietary supplementation commences.[33]

The repression of other anaerobes bacteria, may lead to a kind of adaptation of the intestinal track to more toxic bacteria thus avoiding gastric problems; this hypothesis is evidenced because new-born fed with only infant formulas develop a complex pull of bacteria, which led them to present cases of diarrhea. The bacteria groups included *Streptococcus*, Bacteroides and *Clostridium*, in addition of members of the genus *Bifidobacterium* [33]. On the contrary, breast-fed infants that only presented Bifidobacterium in their GIT presented normal health.

Bifidobactectirum also plays an important role in the adult health. Bifidobacterium gender represents from 3-6% of the adult fecal flora and is considered as key commensals that promotes a healthy gastrointestinal tract (GIT).[28]

Bifidobacteria spp and *Faecalibacterium prausnitzii* are considered beneficial for ileal Crohn disease [34], although the supporting evidence for these benefits remain incomplete [35] the reduction of these bacteria has been associated with the postoperative recurrence of ileal Crohn disease.[36]

As the composition of the gut microbiota is deeply influenced by the diet of the infant[32] the diet can have a major influence on the microbial community composition both in short and long term,[37] thus opening up new possibilities for health manipulation via diet, as proposed by Sokol,[34] who suggested to counterbalance the dysbiosis using *F. prausnitzii* as a probiotic acting like a promising strategy to treat Crohn Disease.[34]

Bifidobacteria, in general, is a genus well adapted to the gastrointestinal tract: In 2002, Schell et al determined a 2.26 Mb genome sequence of an infant-derived strain of *Bifidobacterium longum*, where from the 1 730 possible coding sequence, organized in a 60%-GC circular chromosome, many proteins appeared to be specialized for catabolism of a variety of oligosaccharides including glycoside hydrolases acting on "non-digestible" plant polymers or host-derived glycoproteins and glycoconjugates.[38]

Glycoside hydrolases (GH) help *Bifidobacterium* genus to break carbohydrates to smaller units, and then this sugars can take various biochemical roots. Many of these enzymes catabolize oligosaccharides and come from genes that appeared to have arisen from gen duplication or horizontal acquisition.[28]

Bifidobacteria is one of the main bacteria sold for human consumption as "probiotic" due to their safety record and potential health benefits.

1.4.3 Importance of just FFase to produce FOS

A fructofuranosidase (FFase) possesses hydrolytic activity on low sucrose concentration and transfructosylating activity on high sucrose concentrations.[18] On the other hand, Endoinulinase acts randomly and hydrolyze internal linkages of inulin $(GF)_n$ to yield FOS.[18]

The fructosyltransferases (FTase) hydrolyses the terminal non-reducing β-D-fructofuranoside residues from beta-D-fructofuranosides[39] and possesses transfructosylating activity. The Systemic name of the FFase is beta-D-fructofuranosidase fructohydrolase,[39] it is also called invertase, glucosucrase, beta – fructosidase, fructosylinvertase.[39] In sucrose, fructofuranosidases cleave the β-1,2 linkages and eventually may transfer the fructosyl group to an acceptor molecule such as sucrose and FOS thereby releasing glucose as a by-product.[40,41]

1.5 Glycoside hydrolase family 32

Based on the aminoacid sequence, the β-fructofuranosidase from *Bifidobacterium adolescentis* ATCC 15703 (BaFFase) was classified as a member of the family GH 32, that is included in the GH-J clan (with family GH 68).[42] Clan GH-J have a common β-propeller catalytic domain, with 5-fold repeat of blades, that encloses a negatively charged catalytic pocket consisted of three conversed aminoacids, located within the axial part of the beta-propeller.[43]

The distinguishing feature of clan GH-J is the presence of a 5-fold β-propeller catalytic domain consisting of 5 antiparallel β-strands with a classical "W" topology [42]. The lectin Tachylectin-2, a 236 aminoacid protein from the large granulates of the hemocytes of the Japanese horseshoe crab (*Tachypleus tridentatus*) was the first protein discovered, in 1999, to have a five-bladed beta-propeller[36] (figure 3).

However, different from other GH families that compose clan GH-J, such as GH68, theGH32 family shares another feature: the presence of an additional β-sandwich domain appended to the catalytic β-propeller domain[42] (figure 3).

Although glycoside hydrolases from family GH 32 display a great quantity of protein conformation and substrate specificity,[43] such as invertases, inulinaes, levanases, etc.[44] They share a common feature: the presence of three strictly conserved acidic residues in their catalytic pocket.[45]

GH32 includes 4,381 enzymes belonging to various organisms such as Archea (16), bacteria (3317), Eukaryota (1130) and Viruses (2). Even that the number of GH32 enzymes is enormous, there are only 49 accessions from GH 32 members in PDB (Protein Data Bank),[30] and CAZy (Carbohydrate-active Enzymes)[46] reports only 14 three-dimensional entries from GH 32 enzymes (data according to http://www.cazy.org/GH32.html, updated July 2016).

Structural homology searches for this β-sheet domain by the DALI server[47] found similarities with lectins, which are proteins that possess at least one non-catalytic domain that binds reversibly to a specific mono or oligo-saccharide.[48] Although the exact function of this module is still unclear, Altenbach et al.[49]demonstrated that it is essential for overall protein stability.

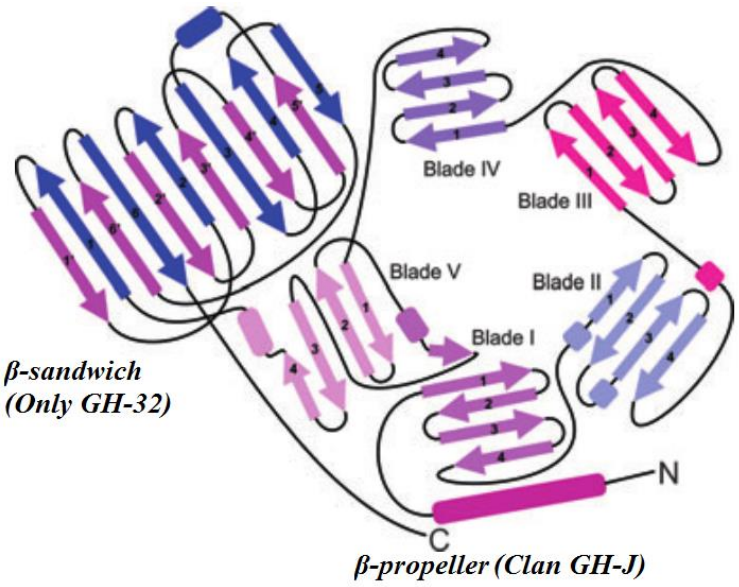

Figure 3 - Clan GH-J and GH 32 general topology.

Source: Adapted from BUJACZ et al.[50]

Bacterial fructosyltransferases catalyze two different reactions depending on the nature of the acceptor: transfructosylation when a fructan is used as the fructosyl acceptor, or hydrolysis when water is the acceptor.[51]

1.5.1 General mechanism of β-fructofuranosidases

GH 32 acts by a retaining mechanism where an aspartate located close to the N terminus acts as the catalytic nucleophile and a glutamate acts as the general acid/base catalyst [42].

Beta-fructofuranosidases catalyzes the release of a β-fructosyl unit from various β-D-fructofuranoside substrates, such as sucrose, 1-kestose, Nystose.[52] Eventually, it may link one or two fructosyl moieties to the sucrose skeleton, forming fructooligosaccharides (FOS). Levansucrases,[53] inulosucrases, and many β-fructofuranosidases can link fructose units by either β(2-1) (1F-FOS) or β(2-6) (6F-FOS) bonds. In contrast, other β-fructofuranosidases such as the β-fructofuranosidase from the yeast *Xanthophyllomyces dendrorhous* transfer the fructosyl moieties to the glucose units of sucrose thus forming the neoseries short-chain FOS.[54]

1.5.2 Active site of β-fructofuranosidases

GH32 β-fructofuranosidase operates with a mechanism with retention of configuration, which will be explaind using the hydrolysis of a raffinose molecule by a β-fructofuranosidase from *B. longum* (figure 4).[50] These three residues constitute part of the highly conserved sequential motifs: NDPNG, RDP and EC, residues that are conserved in yeast, bacterial, and plant invertases as well as on exo-inulinase and fructosyltransferases.[55] The mechanism of retention involves two steps:

First, a nucleophile attack is performed on the anomeric carbon of the sugar substrate by the carboxylate of the Asp 54 acting as the primary nucleophile, forming a fructose-enzyme intermediate. A proton is donated by a Glu 235 to the Glycosyl leaving group.

In the second step (deglycosylation), a water molecule guided by Glu (235) performs a nucleophile attack on the anomeric carbon of fructose (figure 4).

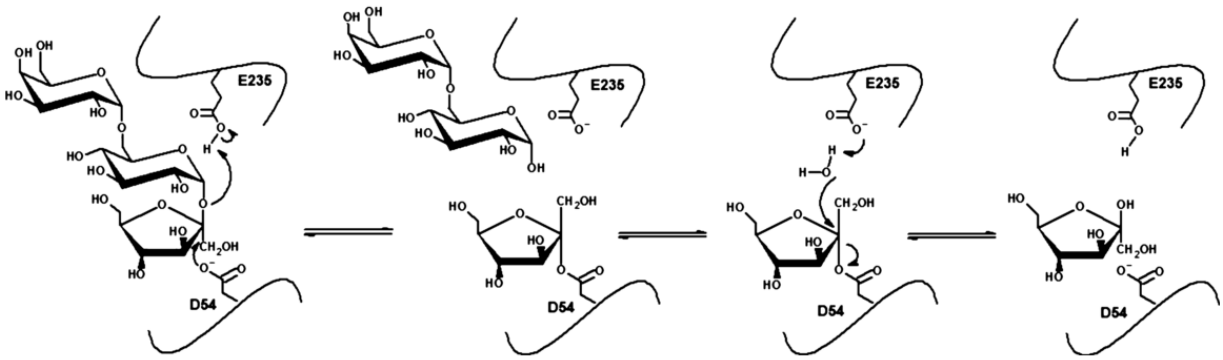

Figure 4 - Double displacement mechanism of reaction from a β-fructofuranosidase that hydrolyzes raffinose.

Source: Adapted from BUJACZ et al.[50]

Three level of controlling substrate specificity have been described by Bujacz.[50] The first deals with the form of the active site pocket. The active site is narrowest and deepest in the yeast and fungal enzymes. The plant enzyme´s active site pocket is the widest and not so deep whereas in the bacterial enzymes it is deep and moderately wide.[50]

The second level, especially for long polysaccharides, is glycosylation, which occurs in fugal, yeast and plant enzymes. The third level of control, found only in the yeast and fungal enzymes, is the dimerization.[50] These three level control the substrate specificity of β-fructofuranosidases.

2 JUSTIFICATION

Bifidobacterium adolescentis is the first bacteria to colonize the gastrointestinal tract of the human new-born, giving him the adaptability previous to colonization by more toxic bacteria and improving the immune system. Bifidobacteria can exert its highly demonstrated benefits for human heath because is able to process non-digestible fructooligosaccharides that we humans consume. To hydrolyze those FOS, these bacteria use a pull of enzymes, among them highlights a set of enzymes called β-fructofuranosidases. Most of these enzymes belong to the GH32 and GH68 family. Even that the number of GH 32 enzymes is enormous, there are only 14 three-dimensional entries from GH 32 enzymes and for *B. adolescentis* ATCC 15703 there is only one crystal structure related to the processing of non-digestible sugars, a xylulose kinase, and no report have been done for β-fructofuranosidases for this bacteria.

In this research, we are going to increase the knowledge of GH32 structure and function by studying the structural and functional features of a recombinant β-fructofuranosidase from the bacteria *Bifidobacterium adolescentis* ATCC 15703. Additionally, our study is going to improve the knowledge of the processing of non-digestible sugars by this bacterium.

3 OBJECTIVES

3.1 General objective

To perform a biochemical and structural characterization of a recombinant β-fructofuranosidase from the bacteria *Bifidobacterium adolescentis* ATCC 15703 heterologously expressed in *Escherichia Coli* Rosetta.

3.2 Specific objectives

- To express BaFFase in the soluble fraction of the *E. coli* Rosetta lysate.
- To purify BaFFase by Ni ion affinity chromatography and molecular exclusion chromatography.
- To test various crystallization conditions and optimize then still grow a difractable crystal.
- To find the optimum conditions (pH and temperature) of hydrolyses of sucrose by BaFFase.
- To compare the initial velocities of reaction of BaFFase when assayed with sucrose, 1-kestose and nystose.
- To reduce the diffraction patterns, solve the structure by molecular or experimental replacement and refine it.
- To analyze and discuss the structure of BaFFase and relate them with their catalytic properties.

4 MATERIALS AND METHODS

4.1 Enzyme production and purification

4.1.1 Chemicals

Sucrose, 1-kestose, Nystose, Tris, NaCl, Imidazol were purchased from Sigma-Aldrich (Saint Louis, MO, USA). CAPS (N-cyclohexyl-3-aminopropanesulfonic acid) was bought from Synth (Sao Paulo, Brazil). Other chemicals were of analytical grade and were purchased from various commercial sources.

4.1.2 Organism, media, plasmids, and culture conditions.

Dr. César Moisés Camilo cloned the gen BAD_1325 in the vector pET-TrxA using the method Ligation-Independent Cloning (LIC) and screened solubility in a semi-automated way in *E. coli*.[56] 12 enzymes were cloned, including the gen BAD-1325, without the need of restriction enzymes (methodology in more detail in reference 54).[56]The ORF of gen BAD_1325 consisted of 1914 base pairs that coded 637 aminoacids.

E. coli Rosetta was transformed with the ligated pET-TrxA vector by the master student Mariana Z. de Lima, and the positive transformants were selected on LB agar plates containing cloranfenicol (35 µg/mL) and kanamycin (50 µg/mL). The strain harbouring this plasmid was named *E. coli* – BaFFase.

E. coli – BaFFase was grown at 37 °C overnight with shaking at 150 rpm in 5mL of LB medium containing 35 µg/mL cloranfenicol and 50 µg/mL kanamycin. Afterwards, the culture was inoculated into fresh medium and cultivated at 37°C until OD_{600} reached 0.5-0.6. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture at the final concentration of 1.0 mM.

After 20 hours induction at 17°C, *E. coli* – BaFFase was harvested by centrifugation (6 000 rpm, rotor SLC 6 000, 30 min, 4 °C), washed with buffer A (0.05 M Tris pH 7.5, 0.3 M of NaCl , 10% glycerol) and resuspended in the same buffer (pellet obtained from 1L of culture was resupended in 50 mL buffer).

4.1.3 Beta-fructofuranosidase purification

4.1.3.1 First step. Extraction

E. coli – BaFFase in buffer A was supplemented with 0.02 mM PMSF, 1 mM of DTT and 0.2 mg/mL of lysozyme, incubated on ice bath for 30 min and ultrasonicated with *Fisher Scientific* sonifier for 8 minutes, 30 seconds on, 30 off at 20 % output and 40 amplitude. The supernatant obtained by centrifugation (36 000 xg, 40 min at 4 °C) was used as the crude cell-free extract.

4.1.3.2 Second step. Chromatography into an Immobilized Metal Ion Affinity Column

The crude cell-free extract was purified by nickel ion affinity chromatography using a Chelating Sepharose Fast Flow column (GE Healthcare, USA) previously equilibrated with buffer A. The column was washed with 10 column volumes (CV) of buffer A and the bound protein was eluted with 10, 20, 30, 50 and 100% of buffer B (Tris 50 mM pH 7,5; NaCl 300 mM; glicerol 10%, 300 mM Imidazol, 1 mM DTT) in buffer A (total volume of 5 CV). The column was cleaned with buffer C (Tris 50 mM pH 7,5; NaCl 300 mM; glicerol 10%, 500 mM Imidazol) for ulterior uses.

4.1.3.3 Third step. BaFFase-Trx cleavage

The active fractions were mixed and diluted 30 times in buffer D (Tris 0.05 M pH 7,5; NaCl 0.150 M; glicerol 10%). The protein solution was incubated with protease TEV (Tabacco Etch virus) to cleave the Trx tag (1mg of TEV for each 50 mg of protein) for 24 hours at 10 °C. TEV is an aminoacid specific protease that recognizes the sequence E-Xaa-Xaa-Y-Xaa-Q-(G/S). In BaFFase-Trx it recognized E-N-L-Y-F-Q-G thus liberating the TrxHis tag from the BaFFase.

4.1.3.4 Fours step. Second IMAC

After verifying the cleavage of the Trx site from BaFFase-Trx by SDS-PAGE 12%, a second affinity chromatography purification was performed. The protein solution was applied onto a Ni Sepharose High Performance affinity column (GE Healthcare, USA) previously equilibrated with buffer D. As the BaFFase had no longer the Trx-His tag, it passed straight and was collected in the flow through; Trx-His tag was eluted from the column with buffer C. The flow through protein solution was concentrated against a 30 KDa cut-off filter (Millipore, Billericak, MA, USA) still 1 mL before purifying by gel filtration.

4.1.3.5 Five step. Molecular Exclusion Chromatography

The BaFFase presented in the flow through was purified to homogeneity by gel filtration on HiLoad 16/60 Superdex 200 prep grade (Amersham Biosciences, Pistacaway, NY, USA) equilibrated with buffer D. The enzyme was eluted at the linear flow rate of 0.5 ml/min and collected 0.5 mL fractions were used as the purified enzyme preparation for functional and structural assays of BaFFase. All chromatography experiments were carried out at room temperature using an AKTAbasic chromatography system (Amersham Biosciences, USA).

4.2 Protein purity

All the purification steps were analysed by Gel Electrophoresis with a Mini-PROTEAN 3 system (Bio-Rad). SDS-PAGE was performed according to Laemmli method [57]using 5% stacking gel and 10% resolving gel. The molecular mass of BaFFase was estimated by comparison with protein molecular weight markers (LMW-SDS Marker kit, Amersham Biosciences, Piscataway, NJ, USA). When the electrophoresis was completed, the gel was stained with Coomasie Brilliant Blue and decolored with a solution consisting of 40% methanol (v/v), 50% water (v/v) and 10% acetic acid (v/v).

4.3 BaFFase isoelectric point

The pHi value of BaFFase was determined by isoelectric focusing in thin slabs of both polyacrylamide and agarose. The method used to determine marker pI values was based on

the procedure suggested by Laas et al [58] .The pI values (24°C ± 1.5°C) are accurate to ±0.5 pH units for markers with pIs < 6 ± 0.08.

Experimental condition of the gel used: Ampholite PAG plate pH 3.5-9.5%T, 3%C PAA thin layer 1 mm thick, 10 cm distance between electrodes. The gel was fixed for 1 hour in aqueous solution of 10% trichloroacetic acid (w, w, v); equilibrated for 30 minutes in aqueous solution of 25% methanol, 5% acetic acid (v, v, v); stained for 10-20 minutes in 0.1% Coomassie Blue G-250 in aqueous solution of 25% methanol, 5% acetic acid (w, v, v); and distained in aqueous solution of 25% methanol, 5% acetic acid (v, v, v) until background was clear.

4.4 BaFFase molecular weight

4.4.1 Molecular Exclusion Chromatography: Linear regression

Gel filtration chromatography is an established method for determining the size and the molecular weight of proteins. This procedure was carried out using a Superdex 200 HR 10/300 GL column (Amersham Biosciences, Piscataway, NJ, USA).

Molecular weight determination of BaFFase was made by comparison of Ve/Vo of BaFFase to the Ve/Vo of six protein standards of known molecular weight (Ve is the elution volume and Vo is the void volume). The void volume of a given column is based on the volume of effluent required for the elution of a large molecule, in this experiment we used Blue Dextran (2,000 kDa).

The protein markers in powder (1mg) were suspended in MQ water (500 µL), centrifuged at 10, 000g (10min), and combinations of 2 protein markers were applied to the column by time. 669 with 75 kDa, 440 with 43 kDa, and 158 with 29 kDa formed those combinations. BaFFase was the last protein applied to the column.

Having the elution curves, we integrated them, and a calibration curve was prepared by plotting the logarithms of the known molecular weights of the six standard protein versus their respective Ve/Vo (table 1). The procedure for determining molecular weights using gel filtration chromatography was a modification of Whitaker[59] and Andrews.[60]

Table 1 - Molecular weight markers, applied to the Superdex 200 10/300 GL column, used to determine the molecular weight of BaFFase by gel filtration chromatography.

MOLECULAR WEIGHT (DA)	PROTEIN NAME	SOURCE	VENDOR
29, 000	Carbonic Anhydrase	bovine erythrocyte	Sigma Aldrich, C 7025
43, 000	Ovalbumin	hen egg	GE, Healthcare
75, 000	Conalbumin	chicken egg white	GE, Healthcare
158, 000	Aldolase	rabbit muscle	GE, Healthcare
443, 000	Apoferritin	Horse Spleen	Sigma Aldrich, A 3660
669, 000	Thyroglobulin	bovine	Sigma Aldrich, T 9145
2, 000, 000	Blue Dextran	Blue Dextran	Sigma Aldrich, D 4772

Source: By the author.

4.4.2 Dynamic Light Scattering (DLS)

DLS measurements were performed using an ALV/CGS-3 compact goniometer system consisting of a 22 mW HeNe linearly polarized laser operating at a wavelength of 633 nm, an ALV 7004 digital correlator and a pair of avalanche photodiodes operating in pseudo cross-correlation mode. Protein solutions (1 mg/mL), in 25 mM Modified Mc. Ilvaine buffer adjusted at different pH values and containing 150 mM NaCl, were first passed through a 0.22 μm filter (Millipore, USA), centrifuged at 16,000xg for 10 min at room temperature, and subsequently loaded into a 10 mm diameter glass cells and maintained at a constant temperature of 25 ± 1 °C.

The autocorrelation functions reported are based on three independent runs of 60 s counting time. The data were collected and further averaged by employing the ALV Correlator Control software. In each case the hydrodynamic radius (RS) was determined.

4.5 Biochemical characterization

Assays above described were performed on triplicate with the enzyme purified by gel filtration chromatography. The values are given in the means ± standard deviation. Data was fitted using the GraphPad Prism 6.0 software.

4.5.1 The standard reaction

The standard pull was formed by three components: purified BaFFase (10 µL), buffer phosphate pH 6.5 (50 µL) and sucrose (40 µL) mixed to achieve a final concentration of 15.2 nM, 0.05 M and 0.05 M, respectively. The reaction was conducted at 25 °C and stopped, after 4 minutes, by adding 100 µL of alkaline dinitrosalycilic acid reagent (DNS). The DNS plus the enzymatic pull were boiled at 95°C for 5 minutes, cooled to room temperature, properly diluted in MQ water and the reducing sugars liberated from the substrate were quantified monitoring the absorbance at 540 nm.[61]

4.5.2 Effect of pH on enzymatic activity and stability

The activity of the purified BaFFase was assayed for sucrose in modified Mc Ilvaine Universal buffer (Sodium Bibasic phosphate – Sodium citrate – Glycine buffer) 0.05 M (pH 2.0-10) and buffer CAPS (N-cyclohexyl-3-aminopropanesulfonic acid) pH 11.0 under standard reaction condition. For the enzymatic stability, 10 times the standard enzyme concentration were incubated in Mc Ilvaine buffer at various pH (2.0-11.0) at 4 °C, after 24 hours, the solution was homogenized, 10 µL of the protein solution was taken and BaFFase was assayed at standard reaction conditions. The residual activity toward sucrose by BaFFase incubated at pH 6.5 was taken as 100%.

4.5.3 Effect of temperature on enzymatic activity and stability

The activity of BaFFase was measured over various temperatures ranging from 4.0 to 70.0 °C. The reaction was conducted as described in section 4.5.3. For the thermostability, 10 µL of BaFFase plus 50 L of phosphate buffer 0.05 M (pH 6.5) were incubated for 1h in the same temperature range, after getting it used to environmental temperature, 40 µL of sucrose

were added and the residual activity was assayed at standard reaction conditions. The absorbance generated by BaFFase incubated at 4 °C was taken as 100%.

4.5.4 Effect of metal ions and chemical reagents

The effect of metal ions on BaFFase hydrolytic activity was assayed in the presence of $MgSO_4$, $CuSO_4.5H_2O$, $NiSO_4.6H_2O$, ZnCl, $LiSO_4.H_2O$, $AgNO_3$, $MgCl_2.6H_2O$, CsCl, DTT and EDTA at a concentration of 2 mM. The additive was added to the buffer phosphate pH 6.5 containing sucrose (both had a final concentration of 0.05 M). The residual activity was measured at 45 °C under standard reactions conditions, where the activity in the absence of metal ions or reagents was considered as 100%.

4.5.5 Substrate specificity

Substrate specificity was determined by analysing the purified BaFFase´s ability to hydrolyze various saccharides including 1-Kestose, Nystose and Sucrose. The enzymatic reaction was composed of purified BaFFase (3 µL), buffer phosphate pH 6.5 (17 µL) and the corresponding saccharide (10 µL), mixed to reach a final concentration of 15.2 nM, 0.05 M and 0.05 M, respectively. The reaction was stopped by adding 30 µL of DNS and the reducing sugars released by the substrate were detected as described in section 6 (Materials and Methods). The enzymatic activity toward sucrose was taken as 100%.

4.6 Structural features

4.6.1 Crystallization and data collection

Crystallization assays were performed with purified BaFFase at 7.6 mg/mL, concentrated by ultrafiltration using a 30 kDa cut-off filter (Millipore, Billericak, MA, USA). The screening of crystallization conditions was done by high-throughput screening with a Honeybee robot by sitting drop vapour-diffusion method at 20 °C on 96 well plates using 500 nL of protein solution and 500 nL of buffer solution against a well with 40 µL of solution. We used the commercial screenings PEG I, PEG II, Crystal Screen, Index Screen, SaltRx from

Hampton Research and PACT Suite, JCSG+ Suite from Qiagen. Index Screen, Crystal Screen, and PEG I and II, presented many crystals like needles, but in one PEG I condition (C10) with PEG 8K 25% (w/v), MES 0.1M pH 6.5, it grew up hexagonal crystals after 3 days of incubation. Crystals were mounted in cryoloops, soaked in cryosolution containing PEG 8 k 25% (w/v), MES 0.1M pH 6.5, Ethylene glycol 5% (w/v), and flash-cooled in liquid nitrogen.

Diffraction data were collected at Brazilian Synchrotron Light Laboratory-LNLS (Campinas, Brazil). The data sets were processed using the CCP4 suite (Collaborative Computational Program, Number 4, 1994).

4.6.2 Structure determination (includes indexing, structure solution and refinement)

BaFFase data set were reduced with Xia2.[62] The structure was solved by molecular replacement with MOLREP[63] using a hybrid model, created using Mr BUMP, consisting of six β-fructofuranosidases/invertases/transferases belonging to *Bifidobacterium longum* KN29.1 (PDB ID: 3PIJ-A),[50] *Schwanniomyces occidentalis* (PDB ID: 3KF3),[64] *Cichorium intybus* (PDB ID: 2ADD),[65] *Haemophilus influenzae* (PDB ID: 1Y7L),[66] *Aspergillus awamori* (PDB ID: 1Y4W)[67] and *Thermotoga maritima* (PDB ID: 1UYP).[68] The template was prepared with CHAINSAW.[69]

Refinement is still being carried out using the program REFMAC in the CCP4 suite.[70] Manual adjustment and rebuild of the model were performed with the program COOT.[71] Progress of the refinement was validated by R_{free} testing. At the further stage water molecules were included in the model, combined to more cycles of restrained refinements that led to a final R-free = 20.0% for all data set up to 2.44 Å (We are still working on this stage). Coordinates and structure factors of BaFFase structure will be deposited in the Protein Data Bank.

Figures were generated using PyMol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC, www.pymol.org).

4.6.3 Low resolution models of the homodimeric BaFFase with SAXS

Small-angle X-ray scattering (SAXS) data collection. SAXS measurements were performed at the SAXS1 beamline of National Synchrotron Light Laboratory, Campinas, Brazil. BaFFase was measured at different protein concentrations (1 and 5 mg/mL) in 50 mM modified Mc Ilvaine buffer adjusted at different pH values and containing 150 mM NaCl. The samples were loaded into a 1 mm path length cell made of two thin parallel mica windows. The wavelength of the incoming monochromatic X-ray beam was λ= 1.55 Å and the sample-to-detector distance was set at 0.95 m, providing an q interval from 0.015 to 0.45 $Å^{-1}$, where q = 4 πsin(θ)/ λ and θ is half the scattering angle.

Three successive frames of 100s were recorded for each sample to monitor radiation damage and beam stability. Buffer scattering was recorded before each sample scattering. The X-ray patterns were recorded using a two-dimensional CCD detector (MarResearch, USA). The integration of SAXS patterns were performed with the FIT2D software and the curves were scaled by protein concentration. The scattering of water measured on the same sample cell was used to normalize the data to absolute scale.

The radii of gyration (Rg) were determined by two independent procedures: 1) by the Guinier equation I(q) = I(0).exp[(-q2.Rg2)/3], q < 1.3/Rg, and 2) using the GNOM program . The distance distribution function P(r) was evaluated with GNOM software and the maximum diameter (Dmax) was determined. Dummy atom models (DAMs) were calculated from the experimental SAXS by ab initio procedures implemented in DAMMIN program.[72] The low-resolution models were compared with each other by the use of the DAMAVER[73] procedure and the most representative model for the whole set was used. The CRYSOL program was used to generate theoretical scattering curves from tridimensional crystallographic structures.[74] The PISA server[75] was employed to search for biological assemblies for the monomeric crystallographic structure of the GH32.

4.6.4 Analysis of refined BaFFase with various software

4.6.4.1 Sequence alignment against PDB

Alignment of BaFFase against Protein Data Bank (PDB) was performed with program BLAST® using blastp algorithm.[76] The search parameter were the following: Word size, 6; Expect value, 10; Gapcosts, 11,1; Matrix, BLOSUM62 and threshold, 21.

4.6.4.2 Structural alignment against PDB

Structural alignment was carried out at DALI server.[47,77] The Dali server is a network service for comparing protein structures in 3D. First, we submitted the coordinates of BaFFase structure and Dali compared them against those in the Protein Data Bank. Then, we selected the beta-fructofuranosidase with more structural similarity and function and performed a multiple alignment using the same server.

4.6.5 Calculus channels and voids

BetaCavityWeb took pdb format of refined BaFFase, recognized molecular voids and channels for a given spherical probe, and computed their geometrical properties such as volume, boundary area, buried area, etc. The algorithms used by BetaCavityWeb are based on the Voronoi diagram of atoms[78] and its derivative construct called the beta-complex.[79]

The recognized voids and channels were reported in a text-based output and were visualized in a graphical interface.

The output consists of three components: 1) the number of cavities, 2) the atoms contributing to the boundary of each cavity, and 3) the geometric property of each cavity. Computational statistics are also reported. The probe radius of zero corresponds to the cavities existing in the van der Waals molecules. If the probe radius is nonzero, the cavities are those existing in the Lee-Richards (solvent accessible) model.

5 RESULTS AND DISCUSSION

5.1 Purification and enzyme intrinsic properties

5.1.1 First and Second purification

The success of the purification of BaFFase-Trx by nickel ion affinity chromatography and by molecular exclusion chromatography was judged by looking the denaturing SDS-PAGE gel. Figure 5 shows an overview of the process. Lane 2 shows a band corresponding to a protein with an approximate molecular weight of 85 kDa. In lane 3, the band corresponding to this molecular weight is very weak, which shows that BaFFase-Trx became attached to the His-Trap Ni column.

Lane 7 shows a single band with a protein corresponding to a molecular weight of approximately 70 kDa, comparing this lane to the 5th, the protein seem to have lost 14 kDa of molecular weight. The 14 kDa reduction in the molecular weight of the enzyme corresponded to the TrxHis tag cleaved from BaFFase-TrxHis by incubating the enzyme for 24 hours with TEV (Tabacco Erth Virus protease). TEV is a protease that recognizes the aminoacid sequence E-Xaa-Xaa-Y-Xaa-Q-(G/S). In BaFFase-TrxHis, TEV recognized the aminoacid sequence E-N-L-Y-F-Q-G thus liberating the thioredoxin protein plus the 6 His tag added at the N-terminal of BaFFase.

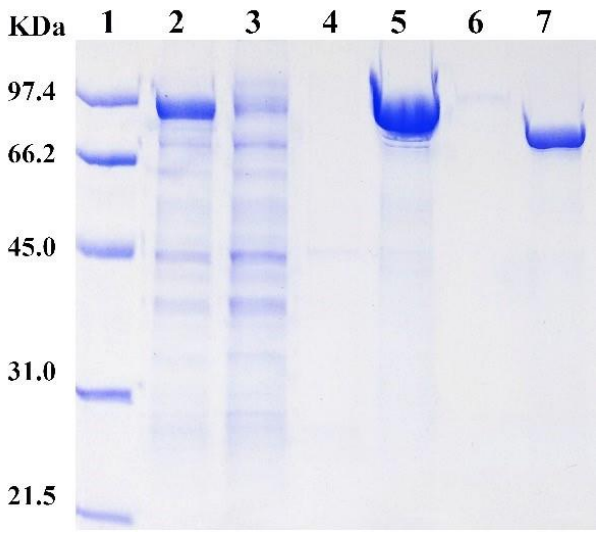

Figure 5 - SDS - PAGE analysis of BaFFase patterns from subsequent purification steps. Lane 1: molecular weight markers; Lane 2: The soluble fraction of E. coli lysate; Lane 3: The Flow through; Lane 4: Equilibrating and washing the His-Trap column with 30 mM of imidazole; Lane 5: BaFFase eluted with 300 mM of imidazole; Lane 6: His-trap eluted with 500 mM of imidazol; Lane 7: The enzyme that passed in the flow through after been incubated with TEV for 24h.

Source: By the author.

5.1.2 Purification by Molecular Exclusion Chromatography and overview of the purification process

During BaFFase purification by Molecular Exclusion Chromatography, we observed a single peak by monitoring the absorbance of each fraction eluted (1mL/frac.). From the 20 fractions comprised within this curve, we decided to collect the ones belonging to the more symmetrical part of it, which were from fraction 57 to 67. Then we run an SDS-PAGE 12% to monitor the pureness of the selected fractions.

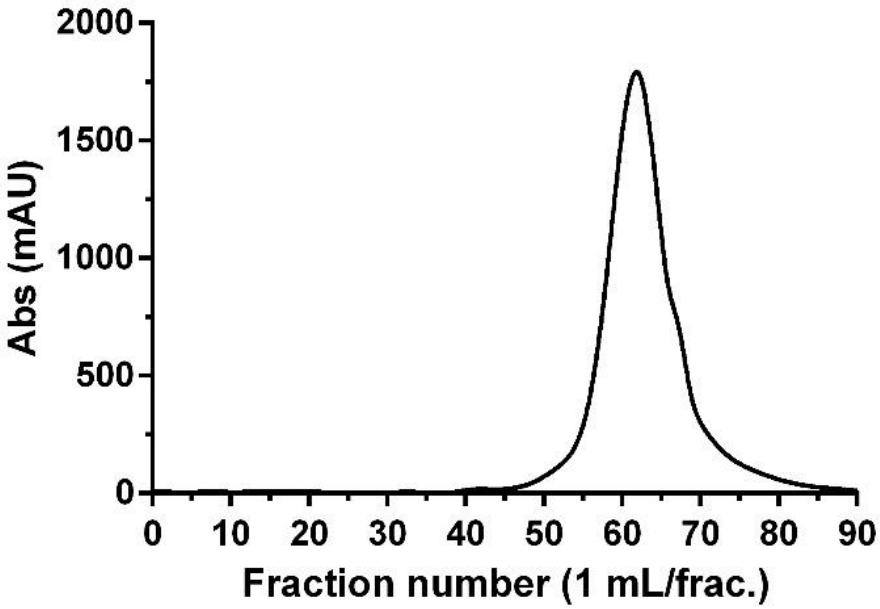

Figure 6 - Purification of BaFFase by Molecular Exclusion Chromatography.

Source: By the author.

Figure 7 shows the fraction of BaFFase when purified by molecular exclusion chromatography. From lane 2 to 10 appear bands corresponding to a protein with 70 kDa. In band 5 and 6 appear to have weak bands with 65 and 60 kDa. These contaminants seem to come from the first purification process, as if we observe the lane 5 from figure 7, one can notice the presence of two weak band, below the one corresponding to BaFFase-Trx, 85 kDa.

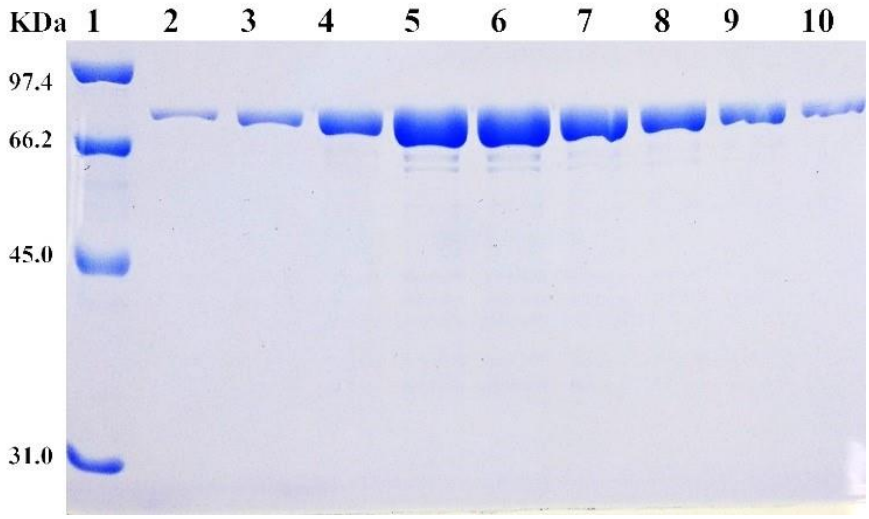

Figure 7 - BaFFase fractions purified by molecular exclusion chromatography.Lane 1: molecular weight marker; Lane 2 to 10: fractions of BaFFase purified by MEC corresponding to the bigger absorbance curve.

Source: By the author.

5.1.3 BaFFase isoelectric point.

Performing the assays in thin slabs of both polyacrylamide and agarose and following the procedure suggested by Laas et al[58] we found that the isoelectric point of BaFFase was 3.5 ± 0.5 (figure 8).

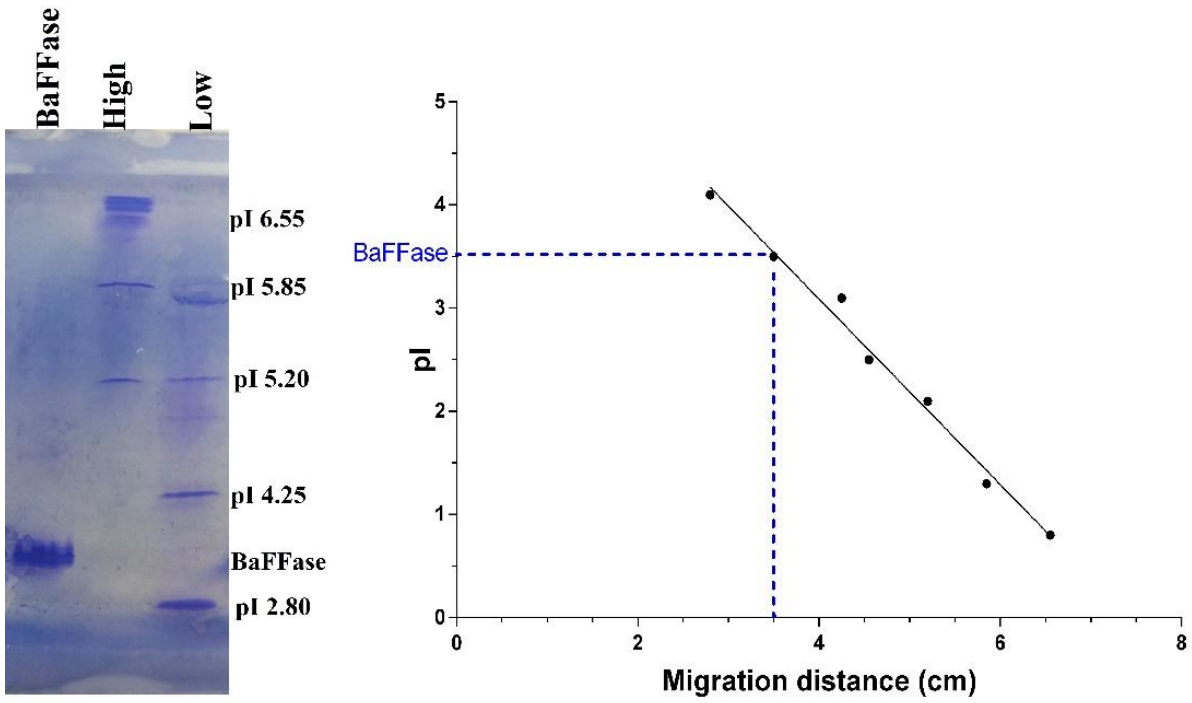

Figure 8 - Isoelectric point of BaFFase assayed in Ampholite PAG plate pH 3.5-9.5%T.

Source: By the author.

5.1.4 Molecular weight of BaFFase

5.1.4.1 *By Molecular Exclusion Chromatography: Linear regression*.

The integrated peak of elution for each protein was the following: Blue Dextran 2 000 kDa, 9.94mL; Thyroglobulin 669 kDa, 11.18 mL; Apoferritin 440 kDa, 12.50 mL; Aldolase 158 kDa, 14.50 mL; Conalbumin 75 kDa, 16.05 mL; Ovalbumin 43 kDa, 17.05 mL; Cabonic anhydrase 29 kDa, 18.35 mL, and the purified BaFFase 14.84 mL.

Having the integrated elution curves, we prepared a calibration curve by plotting the logarithms of the known molecular weights of the six standard protein versus their respective Ve/Vo. The procedure for determining molecular weights using gel filtration chromatography was a modification of Whitaker[59] and Andrews.[60]

From this procedure we obtained that the molecular weight of BaFFase filtrated in the Superdex 200 HR 10/300 was 132.74 kDa. This molecular weight let us see the behaviour of the protein in not denaturing conditions, and taking on count that BaFFase band molecular weight was approximalty 70 kDa, the same as the weight based on the aminoacid sequence;

we supposed that BaFFase was a dimer in solution. To test our hypothesis, we performed DLS measurements, and later we generated low resolution models of the homodimer.

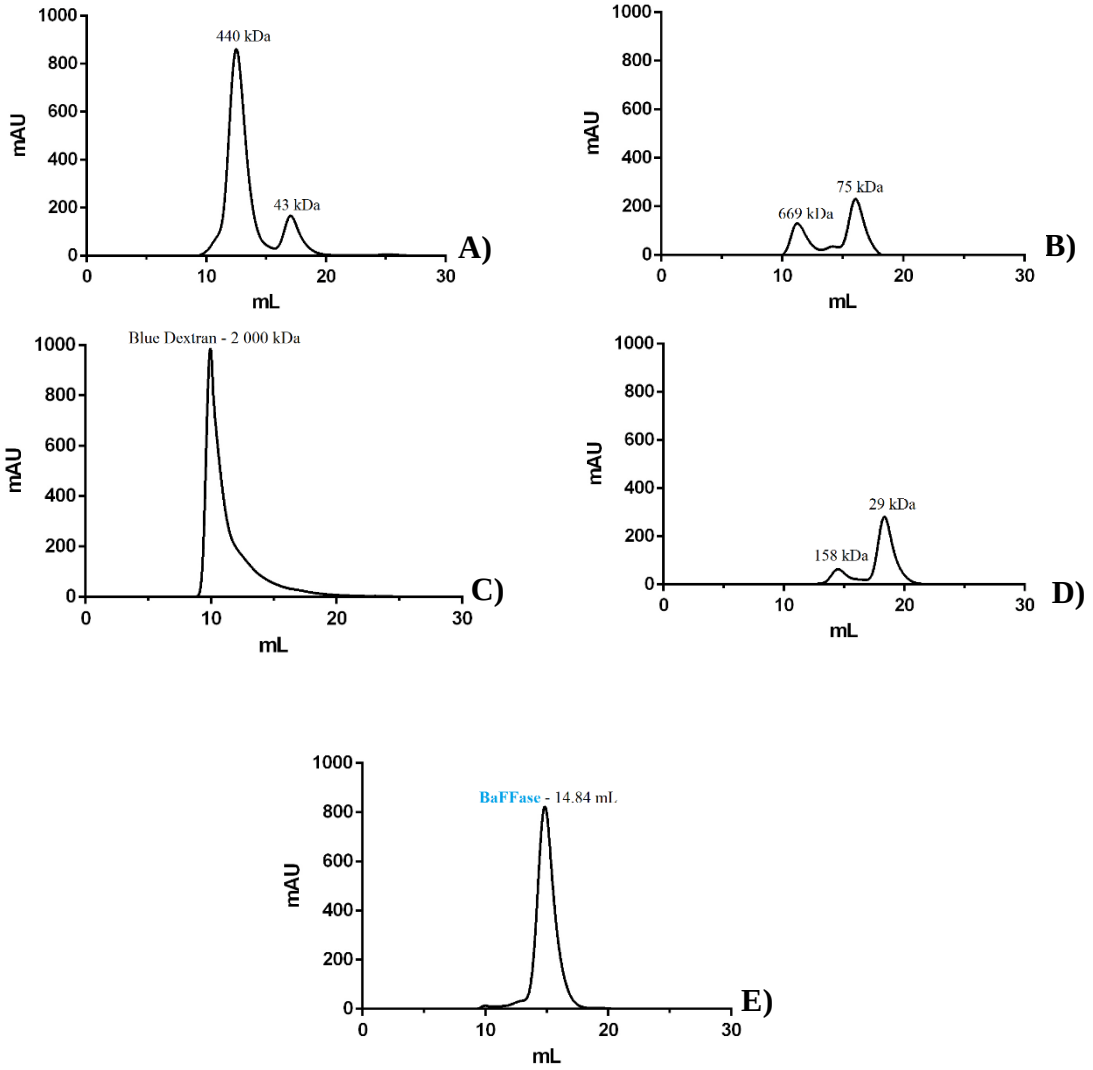

Figure 9 - BaFFase Purification by GFC. The graph plots fractions eluted by gel filtration chromatography versus absorbance at 280 nm.

Source: By the author.

5.1.4.2 *Oligomer state of BaFFase revealed using Dynamyc Light Scattering (DLS)*

To obtain structural information about the tertiary and quaternary structures of GH32, we submitted the enzyme to DLS and SAXS analysis. The observed profile obtained by DLS was characteristic of a monodisperse solution (Fig. 10), whose major peak (representing more than 95% of the total mass) corresponded to hydrodynamic radius (RS) of 4.9 ± 0.3 nm. This value, based on the assumption of a spherical molecule, corresponds to a molecular mass of 139 ± 13 kDa. Therefore, as the molecular mass of a monomer is 70 kDa, the results are most consistent with a homodimer for BaFFase in solution. Furthermore, the results indicated that the oligomerization state of the BaFFase do not change when the pH decreases from 8.0 to 5.0 at 20 °C (inset of figure 10B).

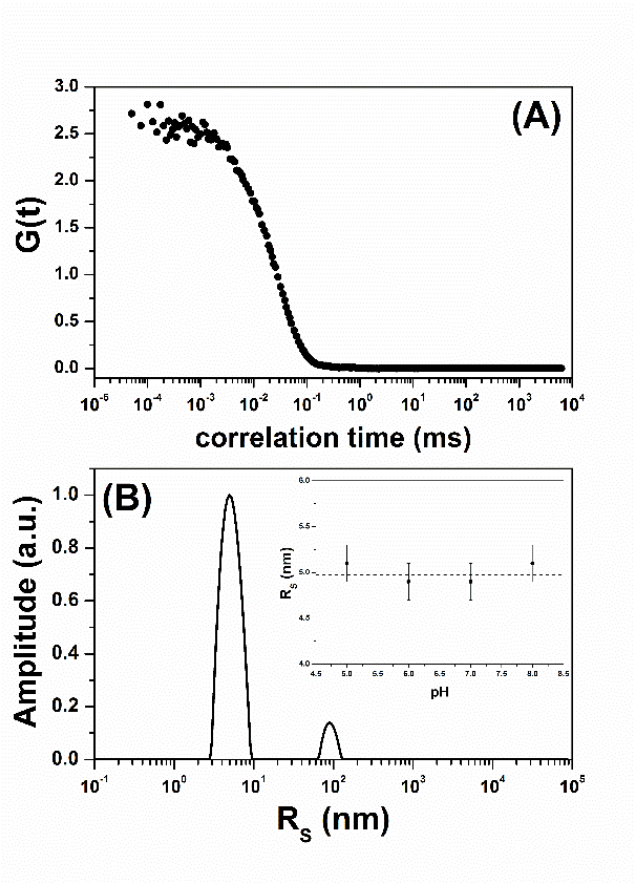

Figure 10 - Dynamic light scattering measurements. (A)Correlation time (ms) in function of the G(t). B) BaFFase Hydrodynamic range in function of the amplitude (a.u.); inset of B) Hydrodynamic range of BaFFase at various pH values (5.0 to 8.0).

Souce: By the author

5.1.5 Discussion from purification and enzyme intrinsic properties

We found that the recombinant BaFFase showed to have an estimated molecular mass of 70 KDa by denaturing gel, that agrees with the size calculated based on the primary sequence, and around 140 KDa when calculated with Gel filtration calibration and DLS. Gel filtration calibration gave us the suspicions of been treating with a dimer β-fructofuranosidase as we obtained 132 kDa as the molecular mass of the peak where BaFFase was eluted, and DLS experiments confirmed the suspicions giving us estimated molecular mass of 139 ± 13 kDa, and SAXS experiment, by using PISA (Protein Interfaces, Surfaces and Assemblies), let us to generate a low resolution model of the dimer.

The contact is made through the N-terminal β-sandwich domain and part of the β-propeller, meanwhile the six- antiparallel stranded β-sandwich does not participates directly in the contact of the homodimer.

Most of the biochemically characterized β-fructofuranosidase from bifidobacteria were monomeric.[50,80–82] Just Imamura, in 1994, described a β-fructofuranosidase from B. infantis JCM No. 7007, as a homotrimer (232 KDa, 75 per monomer);[83] however, to our knowledge, no crystal structure have been presented still now of an oligomer β-fructofuranosidase belonging to the bacteria kingdom.

The molecular weight in solution of β-fructofuranosidases from bifidobacteria genus oscillates among 58-75 kDa. In this line, a β-fructofuranosidases from B. lactis DSM 10140T had 60 kDa,[80] from B. adolescentis G1 74 kDa, [81] from B. longun JCM1217[84] had 59.6 kDa, from B. infantis ATCC 15697.[82] MW stimated was 72 ± 7 kDa; B. longum KN29.1 had 58 KDa.[50]

β-fructofuranosidase from B. longum KN29.1 in solution was in the monomeric form in 99.9 % (also hydrodynamic radius of 16.78 A˚ , average radius of 34.02 A˚ and ratio of the shortest to the longest axis of 0.628).[50]

Many fructofuranosidase from bifidobacteria have molecular weight between 59.4-74 KDa.[80-82,85]

With respect to pHi, we found that BaFFase presented isoelectric point that falls in the acidic pH range. It was 3.54. The isoelectric point of various β-fructofuranosidases from bifidobacteria were from B. adolescentis G1 was 4.5,[81] from B. infantis ATCC 15697 was

4.3,[82] from B. longum KN29.1 was 4.6.[50] Therefore, the isoelectric point of BaFFase was 1 point of pH value lower than other β-fructofuranosidases from bifidobacteria genus.

5.2 Biochemical characterization

5.2.1 The linear part of the curve for 15.2 nM

BaFFase in concentrations of 15.2nM, 152 nM and 304 nM was assayed against 50 mM of sucrose to evaluate its activity (data not shown) with the aim to find a relation that could produce reduced DNS with absorbance lower than 1.0.

Having found that 15.2 nM of BaFFase and 50 mM of sucrose met this requirement, we aimed to find an interval of reaction which absorbance could be located within the linear part of curve sucrose concentration versus time of reaction (figure 11). To perform a standard assay within this linear part facilitates the comparison of the effect of various parameters against the hydrolytic capacity of BaFFase (given as absorbance) as those absorbance values, to be compared, are governed by simple linear equations. Ten minutes of incubation was chosen as the time of reaction for the assays of optimum pH, and pH stability at 25°C, and four minutes (fig.7.) for the optimum temperature, the thermal stability and the metal ions and chemical reagents assays

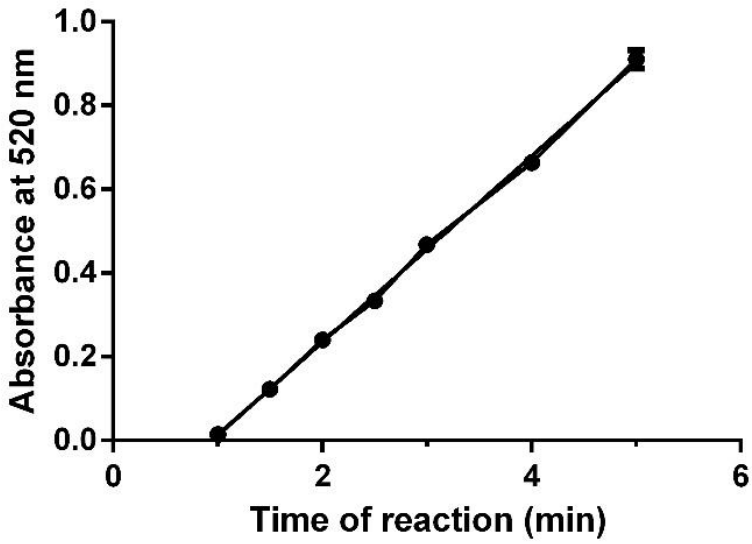

Figure 11 - The linear part of the curve for 15.2 nM of BaFFase incubated with 50 mM of sucrose in buffer phosphate pH 6.5, at 45°C. Four minutes of reaction was chosen for the optimum temperature, the thermal stability and the metal ions and chemical reagents asssays

Source: By the author.

5.2.2 The pH in which BaFFase presents the highest sucrose cleavage.

BaFFase showed to have the best hydrolysis of sucrose at pH 6.5 (figure 12). However, BaFFase maintain a hydrolytic activity higher than 75 % between pH 6.0-7.0 and at pH values lower than 4 and higher than 10 BaFFase activity became almost zero when compared with the activity at pH 6.5.

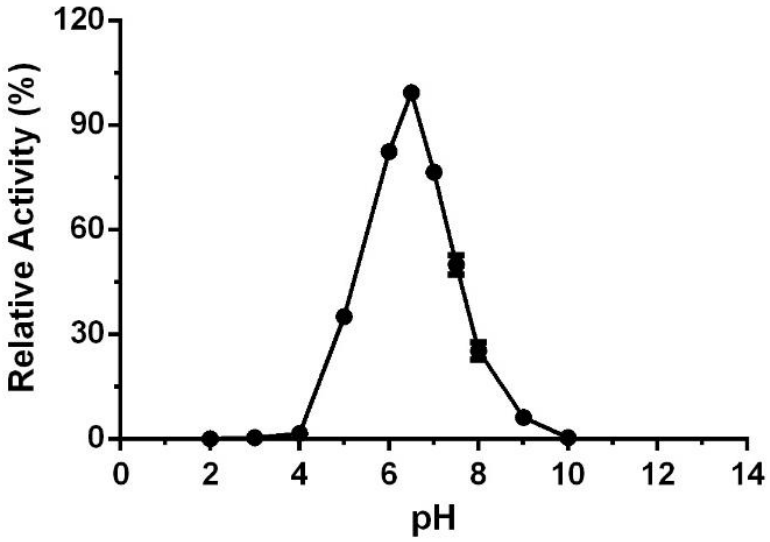

Figure 12 - Effect of pH on the activity of BaFFase. Data given are means ± standard deviations from three replicates

Source: By the author

5.2.3 BaFFase pH stability after 48h of incubation

BaFFase remained stable from pH 6-8 (figure 13) with a hydrolytic capacity higher than 90 %. BaFFase showed to be capable to tolerate especially basic pH, because it remained with up to 70% of enzymatic activity still pH 11.0; however, it manifested lack of activity at pH values lower than 4.0.

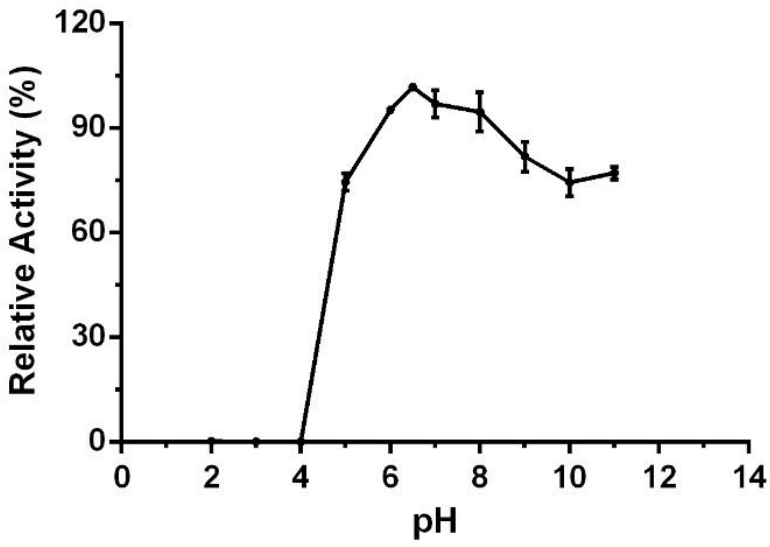

Figure 13 - Effect of pH on the stability of BaFFase. Data given are means ± standard deviations from three replicates.

Source: By the author.

5.2.4 The temperature in which 009 presents the highest sucrose cleavage

The absorbance at 47°C was taken as 100%, and the values were ordered according to this absorbance values. BaFFase exerted a hydrolytic capacity higher than 90% from 45-52 °C, then 45°C was taken as the optimum temperature for subsequent hydrolytic experiments. The enzymatic activity falls sharply from 50°C still becoming almost zero at 60°C, when compared with the activity at 45°C. At 37°C BaFFase shows 50% the activity of the optimum temperature (45 °C).

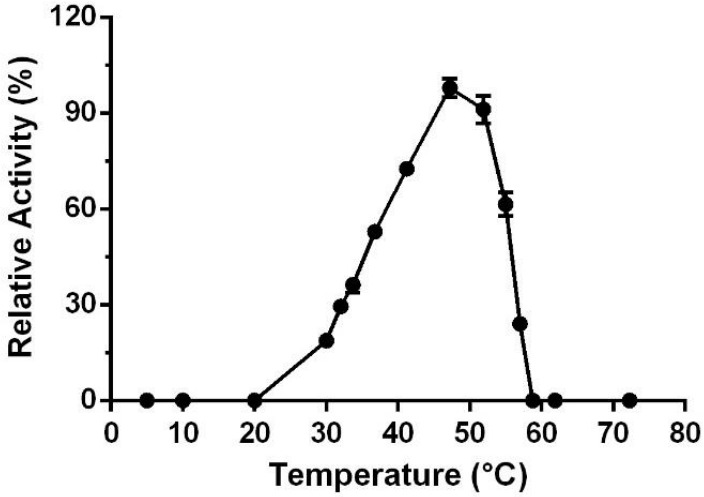

Figure 14 - Effect of temperature on the activity of BaFFase. BaFFase was incubated for 1h at various temperatures (4-72°C) then its activity was measured at standard reaction conditions.

Source: By the author.

5.2.5 The thermal stability for 1h of incubation

BaFFase (10 µL) was incubated in Mac Ilvaine buffer (50 µL) pH 6.5 for 1h at various temperatures, then 40 µL of sucrose with a final concentration of 50 mM was added, and the hydrolytic capacity was tested at standard reaction conditions. The quantity of reducing sugars were taken as indicator of the hydrolytic capacity and the absorbance of the products released by BaFFase stored at 4 °C during 1h was taken as 100 % (figure 15). BaFFase showed activity higher than 95 % between 4-52 °C; from 52°C, the enzymatic activity signed a sharp decrease jumping to 20% at 55°C and becoming almost zero from 60°C onwards.

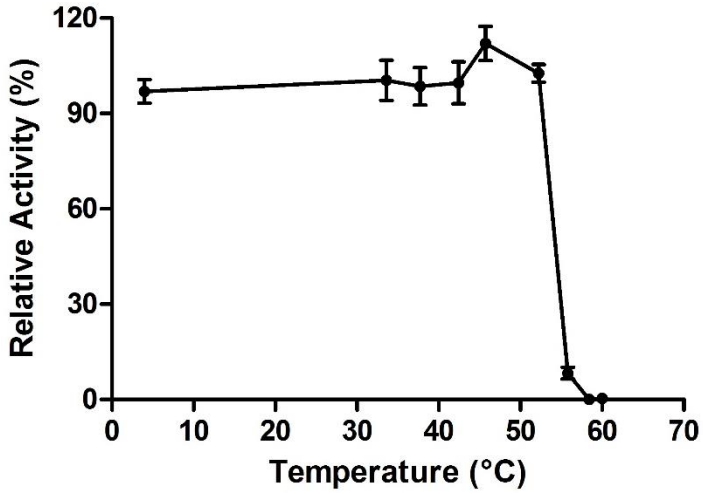

Figure 15 - Effect of temperature on the stability of BaFFase. Data given are means ± standard deviations from three replicates.

Source: by the author

5.2.6 Commentary about the STANDARD REACTION ASSAY

Having discovered the optimum pH, temperature and the pH and thermal stability, we proceeded to assemble the standard reaction assay described in section 4.5.1. Even that BaFFase showed more than 90% activity between 45-52°C, we opted for 45°C as the optimum temperature, and not 52°C, because we wanted to avoid the increase of error measurement. Small temperature variation in the shaker or thermocycler, used to incubate the enzymatic reactions, would affect the measurements as the activity of BaFFase falls down sharply from 52°C onwards. pH 6.5 was chosen as the optimum pH, then the standard reaction assays are carried out in 50mM phosphate buffer pH 6.5.

5.2.7 Activity of BaFFase in presence of metal ions and chemical reagents

The activity of BaFFase was affected by some metal ions (table 2). Cs slightly inhibited BaFFase 84.9 ± 4.5. The presence of Ni reduced the BaFFase activity to 39.5± 6%. In the presence of Cu2+ and Ag1+, the activity of the purified BaFFase was completely inhibited. However, other metals and reagents including Mg 2+, DTT, EDTA had no impact on the activity of BaFFase. Meanwhile, Zn slightly increased its activity by 13.2 ± 7.9 %.

Table 2 - Effect of various metal ions and chemical reagent on the activity of BaFFase.

Reagent	**Concentration (M)**	**Residual activity (%)**
$LiSO_4$	2 x 10 -3	100.0 ± 6
$NiSO_4$	2 x 10 -3	39.5 ± 6
$CuSO_4$	2 x 10 -3	0
$MgSO_4$	2 x 10 -3	88.8 ± 11
CsCl	2 x 10 -3	84.9 ± 4.5
$AgNO_3$	2 x 10 -3	0
EDTA	2 x 10 -3	87.0 ± 2.6
DTT	2 x 10 -3	95.3 ± 1.9
ZnCl	2 x 10 -3	113.2 ± 7.9

Source: By the author.

5.2.8 BaFFase substrate specificity: a comparison of the slopes of Suc, 1-Kes and Nys.

This assay shows that BaFFase was able to release fructose from various sugars: sucrose [β-d-glucopyranosyl-(1-2)-β-d-fructofuranose], 1-kestose [β-d-glucopyranosyl-(1-2)-

β-d-fructofuranosyl-(1-2)-β-d-fructofuranose], and Nystose [β-d-glucopyranosyl-(1-2)-β-d-fructofuranosyl-(1-2)-β-d-fructofuranosyl-(1-2)-β-d-fructofuranose].

BaFFase cleaves 1 – Kestose and Nystose at the same velocity (figure 16). Taking on count the error associated with the DNS detection of the reducing sugars it is difficult to differentiate the capacity of BaFFase to cleave 1- Kestose and Nystose. However, compared with sucrose, BaFFase presents a much higher specificity to cleave 1 – Kestose and Nystose: 18 ± 8 and 9 ± 4 times higher, respectively.

BaFFase as other β-fructofuranosidases catalysed the release of β-fructose from the non-reducing termini of various β-D-fructofuranoside substrates such as the shown sucrose, 1-Kestose and Nystose.

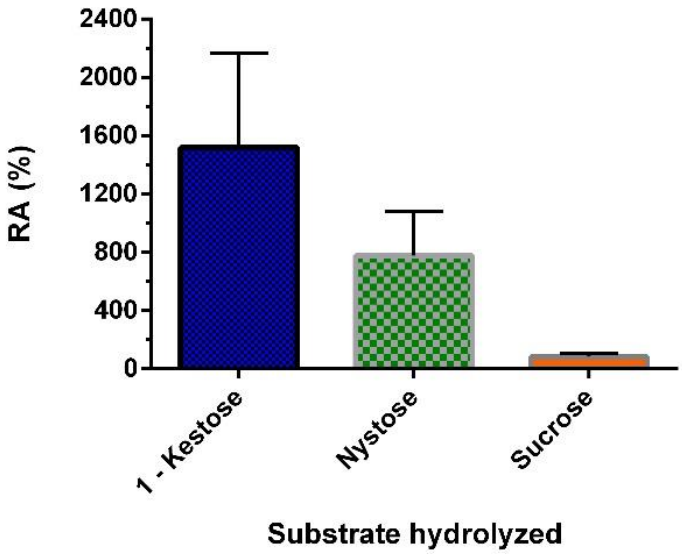

Figure 16 - Capacity of BaFFase to hydrolize 1 – Kes, Nys and Suc. Comparison of the initial velocity of BaFFase to cleave 1- Kestose, Nystose and Sucrose.

Source: By the author.

5.2.9 Discussion from biochemical characterization

We found that the optimum pH of BaFFase was 6.5. BaFFase maintained a hydrolytic capacity higher than 75% between pH values 6.0-7.5, but a single point corresponded to a hydrolytic capacity higher than 95%, pH 6.5, which was taken as the optimum temperature of BaFFase and was used for subsequent experiments. At pH lower than 4.0 and higher than 10.0 BaFFase activity became almost zero when compared with the activity at pH 6.5.

A β-fructofuranosidase from *B. lactis* DSM 10140T[80,85] also had its optimum temperature at pH 6.5. Meanwhile β-fructofuranosidases from other Bifidobacteria genus such as *B. longun* JCM1217, *B. infantis* ATCC 15697, *B. infantis* JCM No. 7007 *B. adolescentis* G1 also had an acidic pH ranging from 5.7 – 6.2.[84,86]

We incubated BaFFase for 24 hours at Modified Mc Ilvaine buffer pH range 2-10 and CAPS pH 10, and then tested the residual activity at standard reaction conditions. We found that BaFFase remained with more than 90% activity between pH 6-8, and with around 70% activity between pH 5.0-11.0, thus showing an excellent tolerance to basic environments. However, it did not occur with acidic pH values as BaFFase manifested lack of activity at pH lower than 4.0.

Starting from pH 6.5, the activity of BaFFase remain with 70% activity up to pH 11.0, and the contrary happens with direction to more acidic pH values, where the activity falls from 90% (pH 6.0) to 75% (pH 5.0) to 0% at pH 4.0.

A β-fructofuranosidase from B. adolescentis G1 showed a similar basic tolerance, it was stable from pH 6.5-10.0 [87], the same happened with a β-fructofuranosidase from B. longum KN29.1 where native and recombinant enzyme were stable in the pH range 5.7-9.1 at 37°C [55]; however, the enzyme was incubated in various pHs for only for 30 min, which is different from the 24 hours of incubation of BaFFase, but at 4°C.

We did not find any β-fructofuranosidases from Bifidobacteria that were very stable at pH values lower than 5.0. On the contrary, β-fructofuranosidase from bifidobacteria gender have been described to be stable between

pH 5-10.[55,80-81,84]

When assayed with sucrose, we found that BaFFase performs its optimal temperature from 45 – 50°C. The optimum temperature of BaFFase was similar to a β-fructofuranosidase from *B. longum* KN29.1[55] (50°C), higher than a β-fructofuranosidase from *B. lactis* DSM 10140T (40 °C),[80,85]and lower than a β-fructofuranosidase from *B. infantis* JCM No. 7007 55°C.[83] However, in general the optimum temperature of β-fructofuranosidases from various genders remain around 50 °C.

BaFFase showed 95 % activity when incubated between 4-52 °C for 1 hour; starting from 52°C the enzymatic activity signed a sharp decrease jumping to 20% at 55°C and becoming almost zero from 60°C onwards.

For ulterior experiments, we used 45 °C as the optimum temperature as BaFFase showed a sharp decrease from 52°C onward, so small changes in the enzyme incubator could have led to increase the measurement error.

We found that BaFFase showed activity higher than 95 % between 4-52 °C; starting from 52°C, the enzymatic activity signed a sharp decrease jumping to 20% at 55°C and becoming almost zero from 60°C onwards.

BaFFase showed to be more stable to higher temperatures when compared with β-fructofuranosidases from the same gender.

Three β-fructofuranosidases were stable still 45 °C: β-fructofuranosidases from *B. adolescentis* G1[81] and from *B. longum* KN29.1.[55] BaFFase had almost the same thermostability for a β-fructofuranosidase from *B. longun* JCM1217[84] than remained stable up to 50°C (activity higher than 90%); however, the last enzyme was inactivated at 65°C, meanwhile BaFFase showed no activity just at 60°C.

BaFFase showed a sharp decrease, so in less than 3°C the stability fell from 95% (52°C) to 20% (55°C). A β-fructofuranosidase from *B. infantis* ATCC 15697[82] also had 20% relative activity at 55°C, but the stability decreasing was not sharp, this enzyme maximal stability was at 37°C, so the temperature fell down to 20% activity only after 18°C.

Enzymes resistant to environmental factors, metal ions, detergents, and organic solvents are highly valued for industrial applications.[88] We evaluated the effect of metal ions and chemical reagents over BaFFase activity and found that its activity was affected by some metal ions.

Cs slightly inhibited BaFFase to 84.9 ± 4.5. Ni presence reduced the BaFFase activity to 39.5± 6%. This result is similar to a β-fructofuranosidase from *B. lactis* DSM 10140T that was inhibited by Ni 2+ but at a concentration of 10mM,[80] compared with the 2mM used for BaFFase assay.

In the presence of Cu^{2+} and Ag^{1+}, the activity of the purified BaFFase was completely inhibited. This result follows the line of Ag^{1+} as a strong inhibitor of β-fructofuranosidase activity found by other authors.[55] Cu^{2+} was considered also as a strong inhibitor of β-fructofuranosidases from B. longum KN29.1[55] and from B. lactis DSM 10140T.[85] The inhibition of Cu2+ over a β-fructofuranosidase from B. lactis DSM 10140T[85] was explained

because of their ability to catalyze the oxidation of thiol groups, which are important for the enzyme activity.

Other metals and reagents including Mg 2+, DTT, and EDTA had no impact on the activity of BaFFase. This results are similar to assays performed with β-fructofuranosidase from B. infantis ATCC 15697[82] and B. longum KN29.1,[55] where neither Fe, Mg, Mn nor DTT, EDTA affected their activity, respectively.

Meanwhile, Zn slightly increased its activity by 13.2 ± 7.9 %. This results are contrary to the reported by Ehrman and Janer for a β-fructofuranosidase from the same strain *B. lactis* DSM 10140T. The first author reported that the enzyme activity was abolished by Zn^{2+} [80] and at half the concentration (1mM) we tested for BaFFase (2mM), and the second found a residual activity of 44 ± 1[85] when the β-frutofuranosidase was assayed in the presence of Zn^{2+}.

BaFFase was able to hydrolyse the β-(1→2) fructosyl between glucose and fructose in sucrose (GF) [α-d-glucopyranosyl-(1-2)-β-d-fructofuranose], and to liberate reducing sugars from 1 kestose [β-d-glucopyranosyl-(1-2)-β-d-fructofuranosyl-(1-2)-β-d-fructofuranose] and Nystose [β-d-glucopyranosyl-(1-2)-β-d-fructofuranosyl-(1-2)-β-d-fructofuranosyl-(1-2)-β-d-fructofuranose].

Based on the mechanism of action of β-fructofuranosidases we could say that BaFFase liberated fructose molecules from 1-kestose and nystose in an exo type even if we only detected reducing sugars by DNS. The hydrolysis of terminal non-reducing β-D-fructofuranoside residues resembles/reaffirms the name of this enzyme as a β-fructofuranosidase.

BaFFase hydrolysed 1-kestose and Nystose 18 ± 8 and 9 ± 4 times faster than sucrose. Thereafter, BaFFase was more efficient to degrade 1-kestose over Nystose, and both of them were more efficiently degraded when compared with sucrose a substrate. Other β-fructofuranosidases from Bifidobacterium were found to degrade FOS over sucrose. Many enzymes shown to have lower hydrolytic capacity against sucrose than 1-kestose.[55,81,83,87] In this line, β-fructofuranosidases from *B. adolescentis*, *B. longum* KN29.1 and *B. infantis* JCM No. 7007 hydrolysed 1-kestose and nystose 3.5 and 2.9,[87] 2.5 and 1.2,[55] 2.9 and 3.6[83] times faster than sucrose, respectively.

The ability of BaFFase to utilize fructooligosaccharides not digested by humans is important for proper bifidobacteria development and function, as well as for symbiosis with their host, which promotes human health.

5.3 BaFFase structural features

5.3.1 Crystal of BaFFase

From the various commercial screenings used, In PEG I and II (Haptom Research), BaFFase formed many crystal like needles, and only in one condition (C10) from PEG I, that contained PEG 8K 25% (w/v), MES 0.1M pH 6.5, it grew up hexagonal crystals after 3 days of incubation (figure 17). Those crystals were mounted in cryoloops, soaked in cryosolution containing PEG 8 k 25% (w/v), MES 0.1M pH 6.5, Ethylene glycol 5% (w/v), and flash-cooled in liquid nitrogen and the diffraction data were collected and processed as described in section 4.6.1.

Figure 17 - Crystals of BaFFase. Crystals of BaFFase in PEG 8 k 25% (w/v), MES 0.1M pH 6.5, condition C10 from PEG Suite I (Hampton Research).

Source: By the author.

5.3.2 General topology of BaFFase

BaFFase presents three domains: a β-sandwich domain and a five-bladed beta-propeller in the N-terminal, and another beta-sandwich domain in the C-term. The N-terminal β-sandwich goes from residues 3 to 93 and presents two β-sheets of four antiparallel β-strands connected by intricate loops. This domain is connected to the β-propeller (blade I) through a loop of 32 residues, which present a helix of 5 aminoacids (residues 107-113).

The β-propeller is the biggest domain, covering 363 aminoacids (residues 127-489), and is composed of five β-sheets located radially and pseudo symmetrically around the central axis. Each β-sheet consist of four antiparallel β-strands connected by loops in a classical “W” assembly, except for the loops 2 from blade III (L2-bIII) and IV (L2-bIV), which present intricate connections with their preceding β-strand: L2-bIII circles the entire blade III before connecting the strand 2 to 3 (bIII), and L2-bIV circles the strand 1, from blade IV, before

connecting to the strand 3. Blade V is connected to the C-terminal β-sandwich through a loop that contains a helix of eight residues.

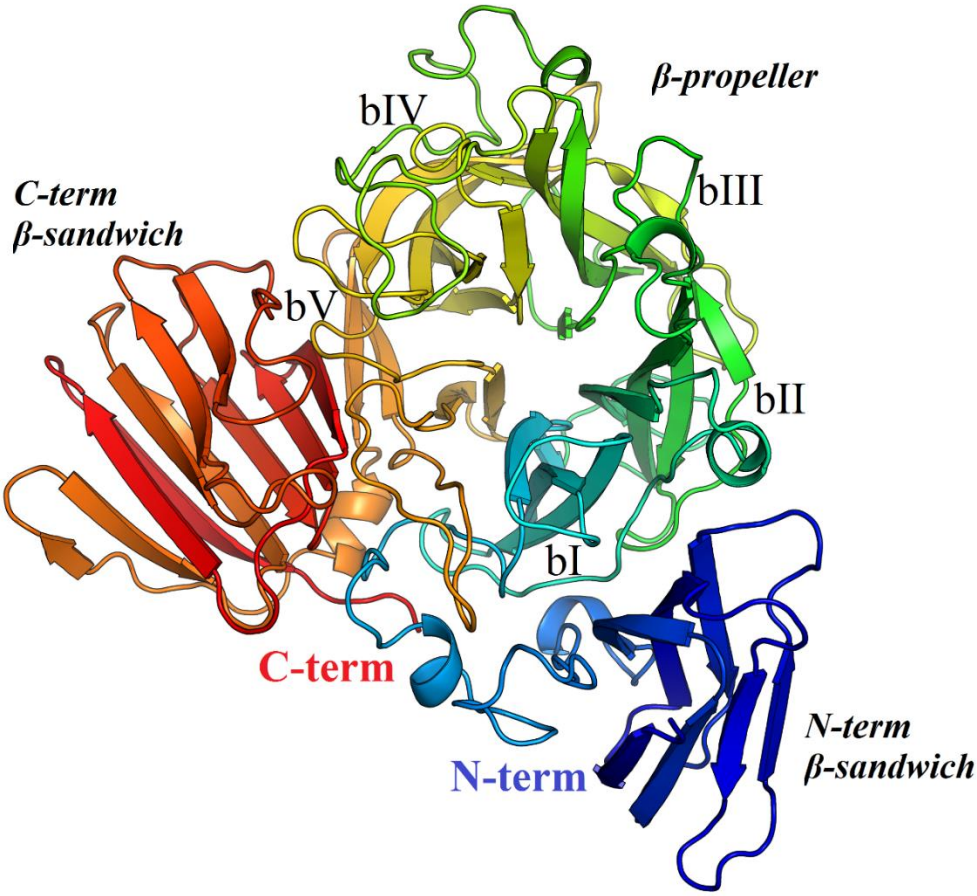

Figure 18 - Ribbon diagram of BaFFase showing the bimodular architecture coloured in rainbow sequence-code, which folds into three domains: an N-term β- sandwich (blue), a catalytic beta-propeller, and a C-term beta-sandwich domain (red). The five blades of the β-propeller domain are represented as bI-bV.

Source: By the author

It is found seven helices along the 633 aminoacids of BaFFase. three helices with just three aminoacids, two with four, one with five and the longer one presents seven residues (Ser491- Leu497) which is located within the loop that connects the β-propeller (bladeV-strand 4) to the C-terminal β-sandwich. From the seven helices, five are located within the β-propeller, where blade V is the only one that lacks a helix in their interblade loops (table 3).

Table 3 - Localization of helices in BaFFase.

No.	Start	End	No. residues	Length	Residues per turn	Sequence
1	Leu85	Asn88	4	5.75	4.04	LLDN
2	Leu108	Tyr112	5	7.53	3.79	LEQIY
3	Pro182	His185	4	7.18	3.22	PELH
4	Glu231	Ser233	3	-	-	ESS
5	Val296	Glu298	3	-	-	VSE
7	Ala303	Asp305	3	-	-	AAD

8	Ser491	Leu497	7	10.85	3.78	SEVYREL

Source: By the author.

The C-terminal β-sandwich domain covers 136 aminoacids (residues 498-633) and is shaped by two β-sheets, each one composed of six antiparallel β-strands connected by loops. This domain lacks helices on its loops.

5.3.3 BaFFase presents an additional β-sandwich not described for other GH32 member

The sequence of the N-terminal β-sandwich (residues 3-93) of BaFFase does not match with any aminoacid sequence of the proteins deposited at PDB database. The alignment was performed under the parameters described in Materials and Methods.

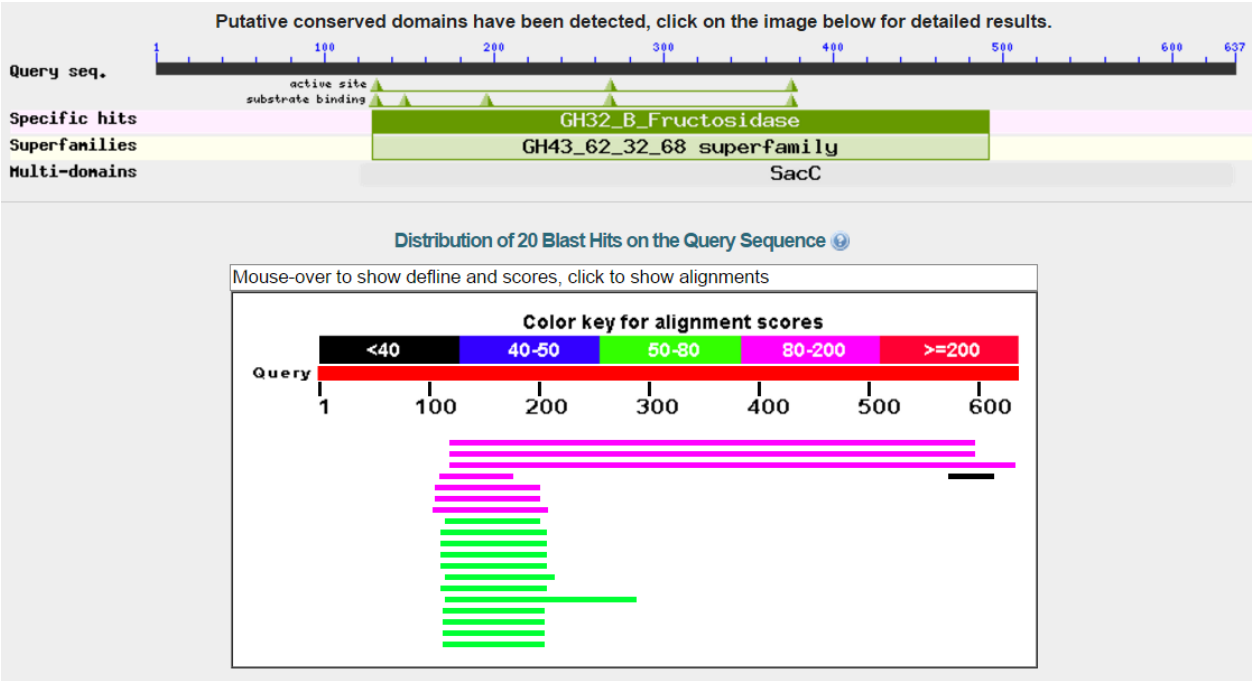

Figure 19 - Alignment of BaFFase aminoacid sequence against Protein Data Bank (PDB) using BLAST. The sequence of the initial 100 aminoacids of BaFFase does not match with any aminoacid sequence of proteins deposited in PDB until July 2016.

Source: Adapted from BLAST.® [76,89]

Beta-fructofuranosidase from *Thermotoga maritime* (PDB ID: 1UYP)[68] had the maximum identity with BaFFase (27%, 130/486 residues) when the sequence of BaFFase was aligned against the PDB database (figure 20).

The most conserved region is the one between residues 115-200 in BaFFase. Within this sequence range, in BaFFase, is situated four strands (β8- β11) from blade I, three strands (β12 – β14) from blade II and a four residues helix (loop that connects strand 2-3 within blade II).

Figure 19 shows the structural alignment of BaFFase against five β-fructofuranosidases from various organisms including yeast, bacteria and plant. The highly conserved aminoacids from the comparison of the five β-frutofuranosidases are **H** (141), **Q** (145), S**GS** (196-198) and **YA** (36-437). Additionally, it is important to highlight three conserved regions implicated in the BaFFase catalysis: (1) **W**M**NDP**N**G**L (residues 128-135), the Asp (D-131) acts as the nucleophile, where 3pijA presents an Ile (I) in the corresponding position Met (M-129), 5annA have a Met (M) instead of a Asn (N-133), and 2xqrA presents a Pro (P) instead of a Leu (L-135). (2) **RDP** (268-270) Asp 269 act as the stabilizer of the transitions state; and (3) ECP (375-377), the Glu (E-375) has been implicated as the acid base catalyst.[54]

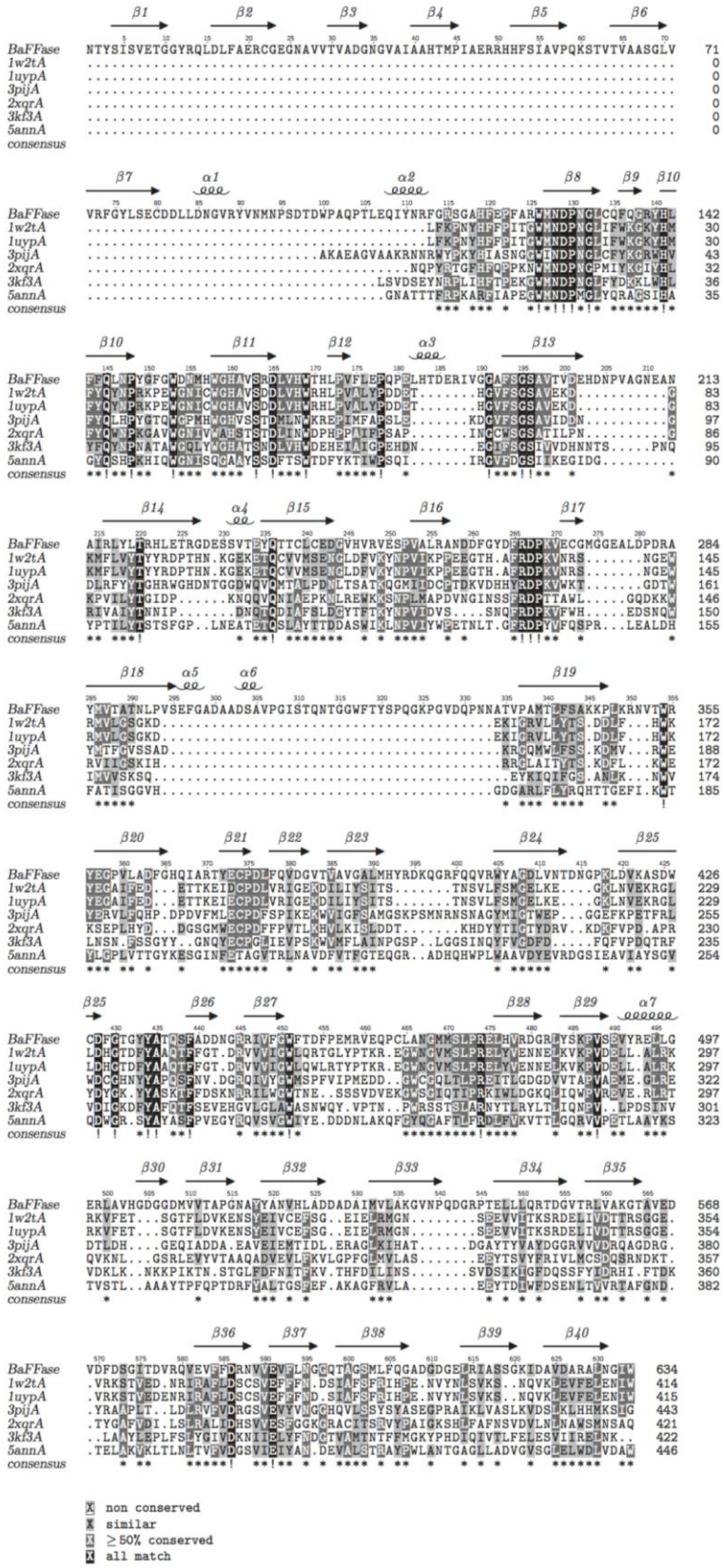

Figure 20 - Structural alignment of BaFFase against five beta-fructofuranosidases structurally closer: 1w2tA (*Termotoga Maritima*), 1uypA (*T. Maritima*), 3pijA (*Bifidobacterium longum* KN29.1), 2xqrA (Arabidopsis thaliana), 3kf3A (*Schwanniomyces occidentalis*).

Source: By the author.

5.3.4 Graphical display of the N-terminal beta-sandwich and its topology

Using Pymol[90] we performed a structural alignment of BaFFase refined structure against 5ann-A from *Xanthophyllomyces dendrorhous*; 2xqr-A, *Arabidopsis thaliana*; 3kf3-A, *Schwanniomyces occidentalis*; 3 pijA, *Bifidobacterium longum*; 1uyp-A, *Thermotoga Maritima*; 1w2t-A, *T. Maritima*. In figure 21, we wanted to highlight the presence of the unmatched 100 aminoacids of BaFFase when compared against PDB (figure 19). Many β-fructofuranosidases usually present a helix before beginning the β-propeller; BaFFase also presents a helix (7 residues) in the loop previous to the β-propeller, however, before this helix BaFFase also presents a β-sandwich domain with two β-sheet composed of four antiparallel β-strands, whose topology will be described in more detail in figure 22.

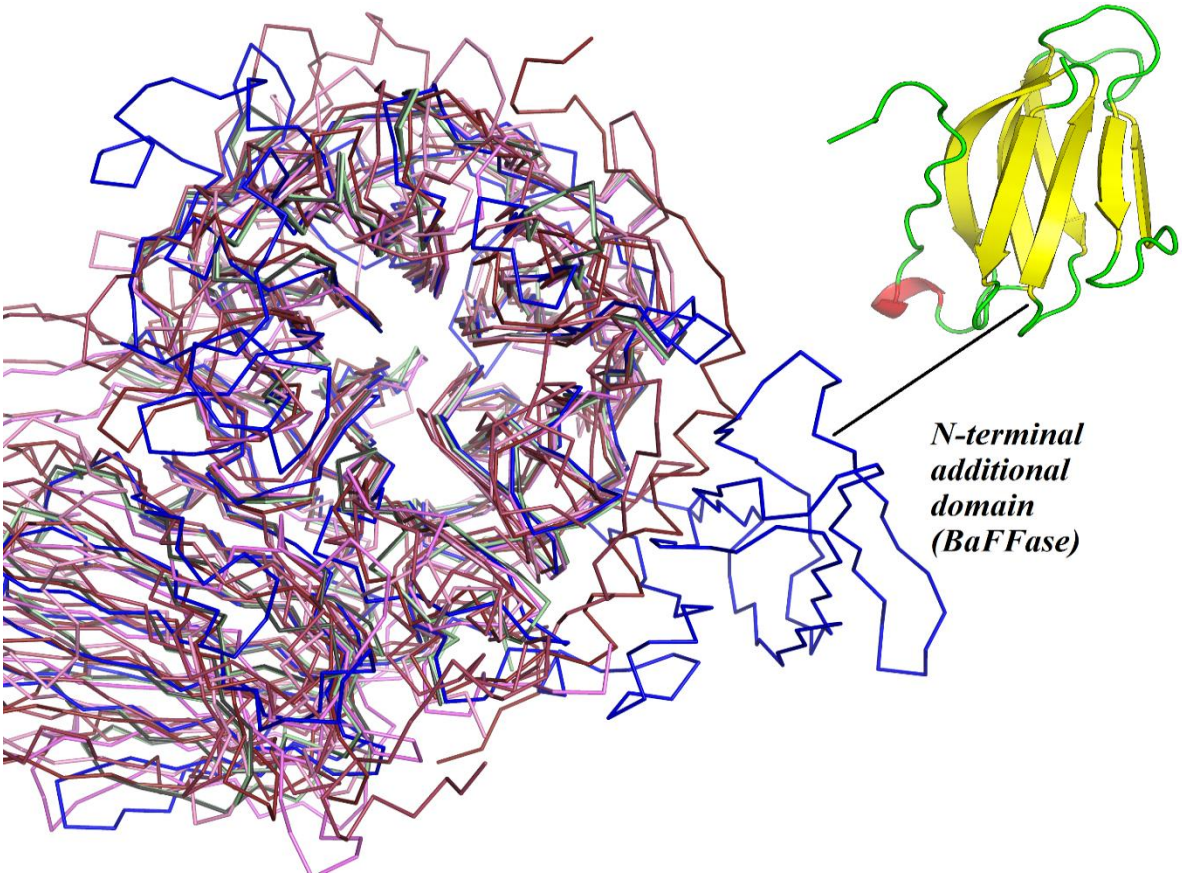

Figure 21 - BaFFase aligned to β-fructofuranosidases structures deposited at PDB. The 100 additional aminoacids from fig.17 shape a β-sandwich. Color codes: BaFFase, blue; 5ANN-A, raspery; 2XQR-A, pink; 3KF3-A, violet; 3PIJ-A, ruby orange; 1UYP-A, palegreen; 1W2T-A, grey

Source: By the author

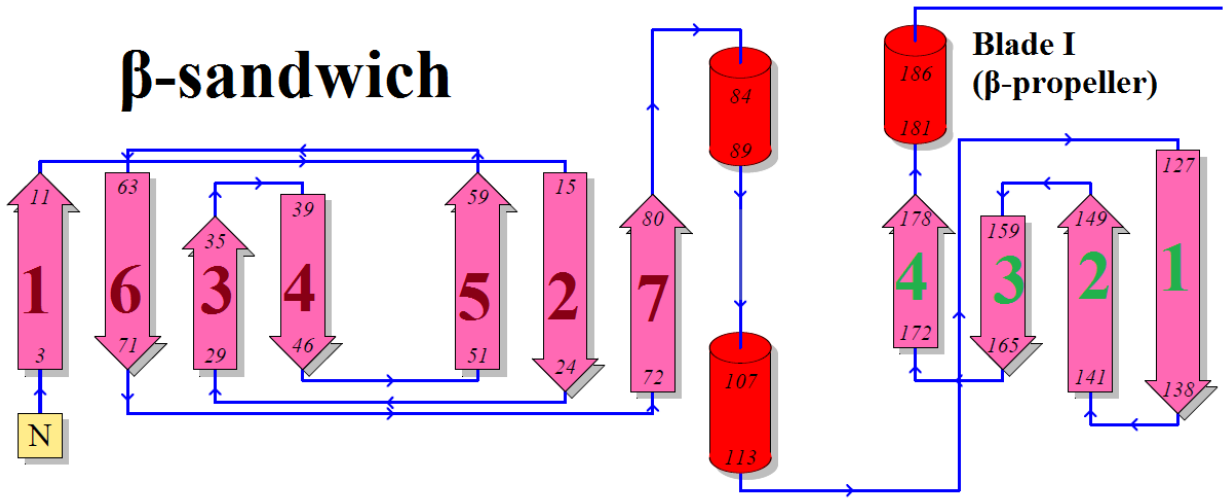

Figure 22 - Topology of the N-terminal β-sandwich. Beta-strands are shown as pink arrows and helices as red cylinders.

Source: By the author.

From aminoacid 3-93 is located a β-sandwich domain composed of two β-sheets with four antiparallel β-strands (figure 22). The seven β-strands are numbered from 1-7 and comprise the following residues: β-strand 1 (resid 3-11), 2 (resid 15-24), 3 (resid 29-35), 4 (39-46), 5 (51-59), 6 (63-71), 7 (72-80), Antiparallel β-strands 1, 6, 3 and 4 form the first β-sheet and β-strands 5, 2 and 7 form the second one. Beta-strands form polar contacts with their antiparallel neighbor strands, but not with the strands located in front of them.

5.3.5 Active site of BaFFase

Many of the strictly conserved aminoacids (comparing the six β-fructofuranosidases described above) are located in chains that shape the active site. The blade I present the higher conserved region.

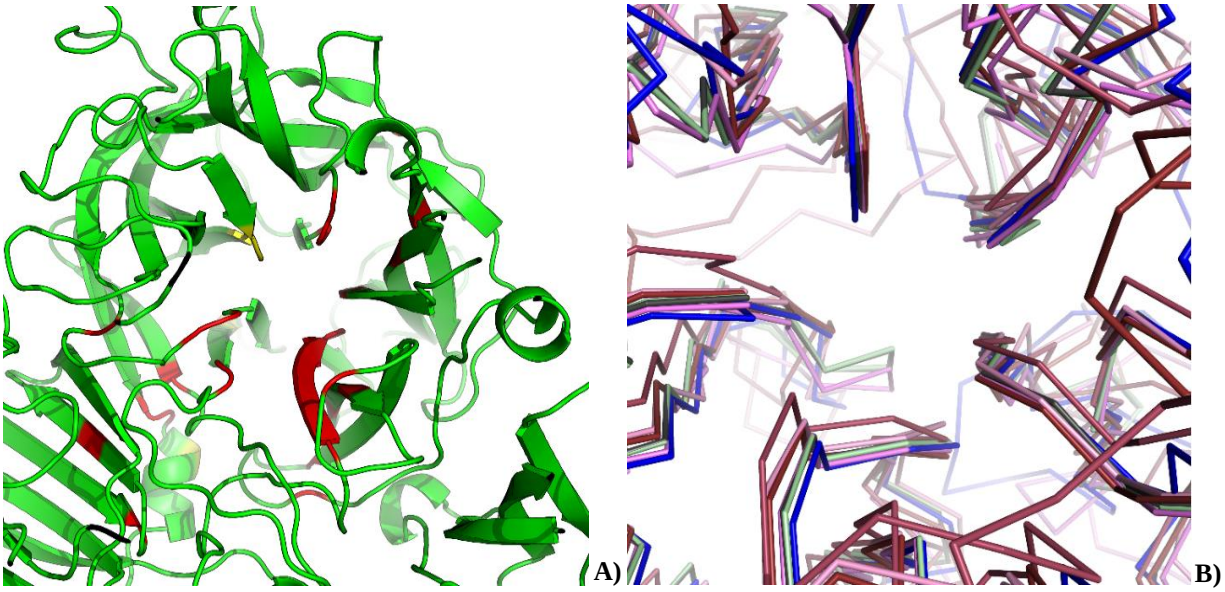

Figure 23 - The aminoacids that shape the active site are the most conversed. BaFFase cartoon diagram where some aminoacids have been coloured according to their similarity level: red, the highest similarity; yellow, one residue of difference. The similarity is based on the alignment shown in figure this -1. (B) A superposition of the catalytic pocket from various β-fructofuranosidases. Color codes: BaFFase, blue; 5ANN-A, raspery; 2XQR-A, pink; 3KF3-A, violet; 3PIJ-A, ruby orange; 1UYP-A, palegreen; 1W2T-A, grey.

Source: By the author

5.3.6 Enzyme 3pijA as a template to deduce the polar contact of fructose with BaFFase

The structure of 3PIJ-A was used as a template to find the active site and the possible polar contact of the deduced FRU location, with the surrounding residues in BaFFase (figure 24).

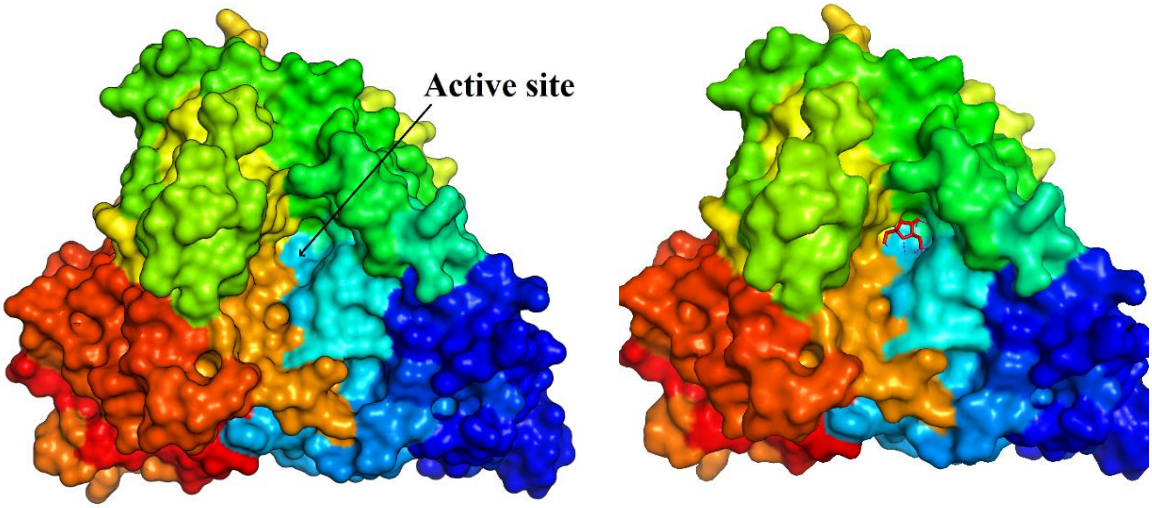

Figure 24 - BaFFase in surface representation pointing the active site. B) BaFFase surface showing the active site and B) in contact with a fructose. The negative active site is located within the axis of the five-bladed β-propeller.

Source: By the author.

A β-fructofuranosidase from *B. longum* (PDB code 3PIJ-A) was used as a template to calculate the location of a fructose and the covalent bonds that fructose could establish with the surrounding aminoacids from BaFFase. Beta-fructofuranosidase 3PIJ-A was selected as template because it presents high similarity with BaFFase around the central axis of the β-propeller and also, because it is an enzyme from the same genre as BaFFase (Bifidobacterium).

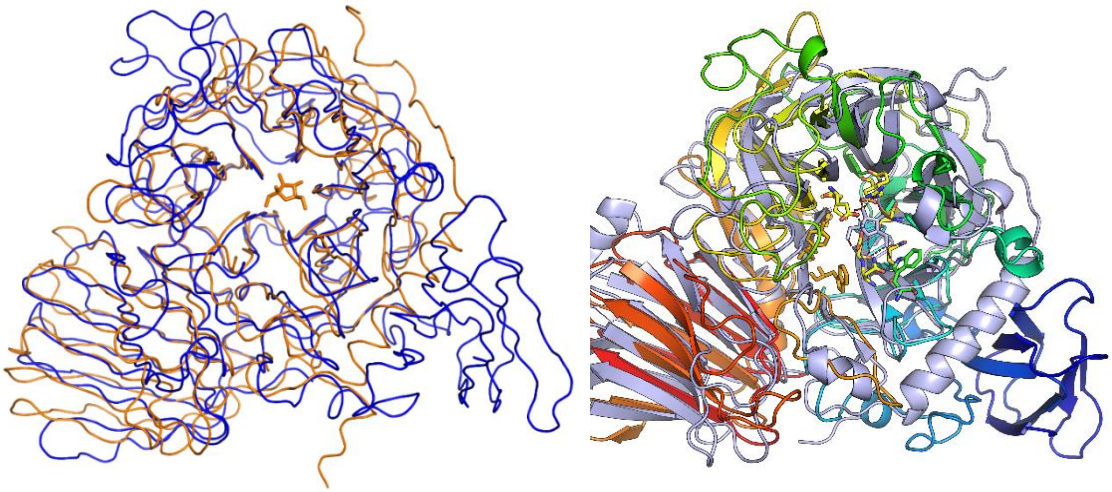

Figure 25 - 3pijA aligned to BaFFase. 3pij is shown in orange and BaFFase in blue.

Source: By the author.

Figure 24 A and 25 left shows the BaFFase catalytic pocked deduced based on the superposition of a β-fructofuranosidase from *B. lomgum* (PDB code 3pij chain A) over

BaFFase and the extrapolation of the position of the FRU (fructose), which is shown in red. Fig. 24B and 25 right shows how it looks the fructose positioned in the catalytic pocket.

5.3.7 BaFFase description of polar contacts in the active site

Figure 26 shows the polar contacts that make the deduced position of fructose with their surrounding aminoacids. Fructose makes 11 polar contacts whose ordered based on the order of their carbons, are the following: with C1: Asp-131, C2: Glu- 375; C3: Glu-375, Arg-268, Asp-269; C4: Asp 269, Ser-196; C6: Gln-147, Trp-155, Asn-130. From the eight residues involved in the polar contacts with FRU, 2 are negative (Asp 131, 269), 1 is positive (Arg 268), 3 are polar uncharged (Asn-130, Gln-147, Ser-196) and 1 is nonpolar, hydrophobic (Trp-155).

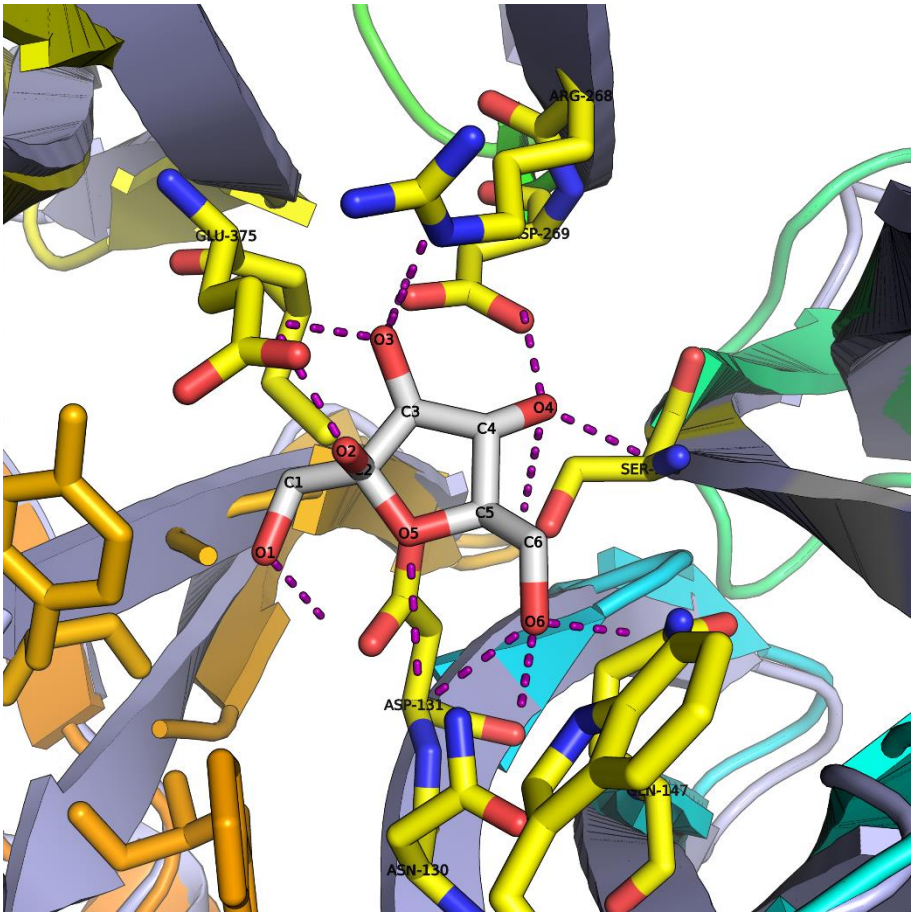

Figure 26 - Fructose establishing polar contact with the aminoacids from BaFFase. Those residues have been identified. Fructose position has been deduced from 3pijA (B. *longum* KN29.1).

Source: By the author.

The active site is well conserved among GH 32 family, consist of three aminoacids. A nucleophile, a stabilizer for the transition state, and the acid/base catalyzer. In figure 27, we show the atoms of each aminoacid involved in the fructose transferring activity.

Residues Glu 375 is the proton donor and Asp 131 acts as the nucleophile. These residues are located as the first β-strand of blades 1 and 4 in the N-terminal β-propeller domain.

Asp (D-269) from the RDP motif establishes two polar contacts with oxygen 3 and 4 from fructose. The D from the RDP motif has been implicated in the intermediate stabilizer.[91]

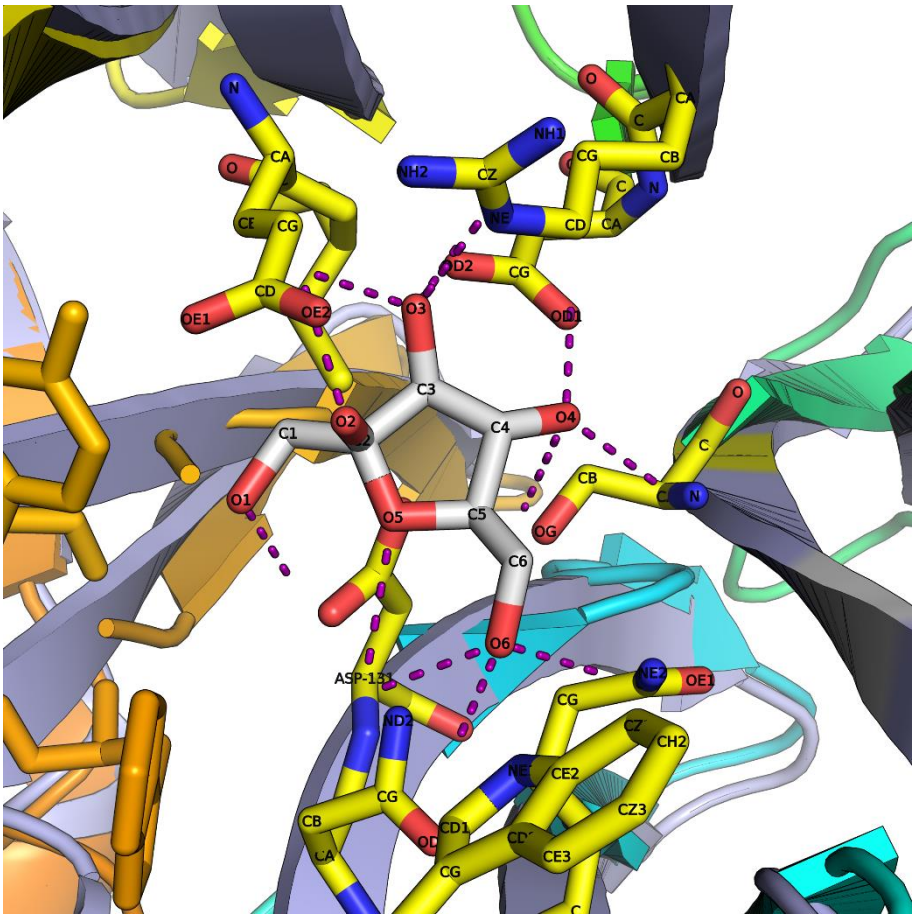

Figure 27 - Polar contacts of fructose with the surrounding BaFFase residues, named by atom. The negative active site is located within the axis of the five-bladed β-propeller.

Source: By the author.

5.3.8 Biggest channels and voids

BetaCavityWeb found seven channels, from them, one has been highlighted (channel A) because describes the catalytic pocket (figure 28). Channel A has two openings and nine

bottleneck. This channel is defined by 61 aminoacids, especially ones between sequence 230-390 approximately.

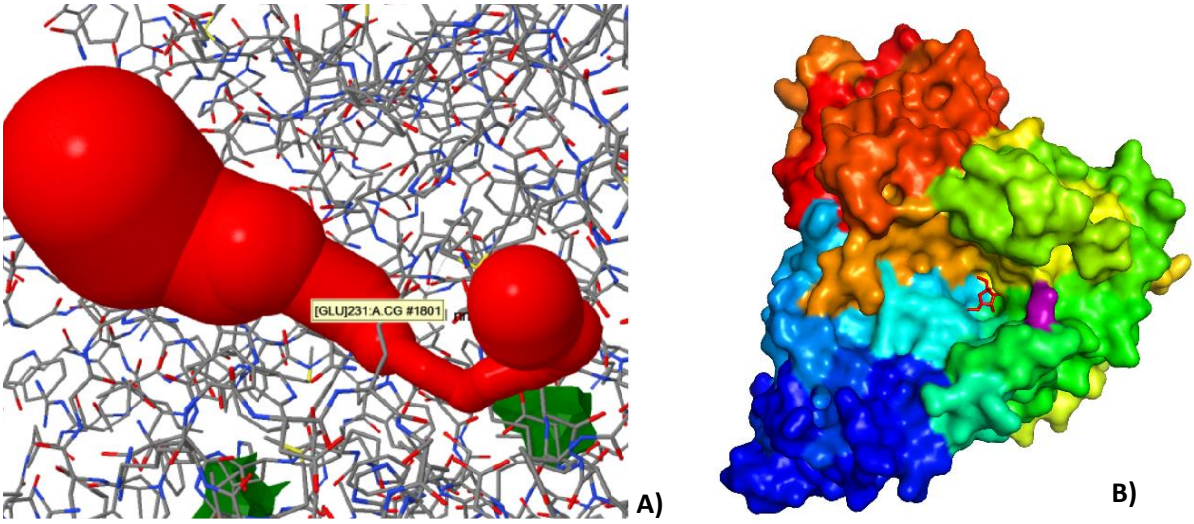

Figure 28 - A) Channel of 61 aminoacids found with BetaCavityWeb. B) Channel view in BaFFase surface representation. The Glu 231 pointed in figure A, is colored as pink in figure B.
Source: By the author.

Beta cavity web also calculated voids in BaFFase structure (figure 29). Void A has 29.4 A3 of volume, 73.3 A2 of area boundary, and 26 atoms define this boundary. Void B, which is located on the opposite site of the channel A, presents 11.1 A3 of volume, 44.7 A2 of area of boundary, and 24 atoms define this void.

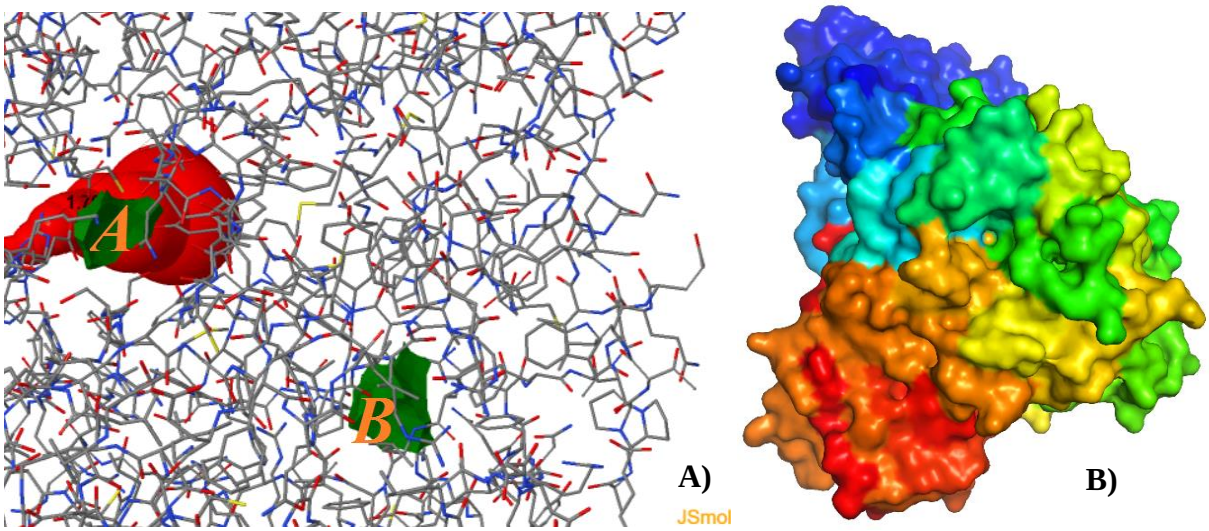

Figure 29 - (A) Voids found with BetaCavityWeb within BaFFase. (B) Show the back part of the BaFFase catalytic pocket in surface representation.
Source: By the author.

5.3.9 Tertiary and quaternary structure of BaFFase with SAXS

To prove that BaFFase is a homodimer in solutionand to obtain information about its molecular shape, we submitted the enzyme to SAXS analysis (Figs. 30 and 31). The X-ray scattering curves of BaFFase obtained at different pH values are shown in Fig. 29. At pH 7.0, the radius of gyration (Rg) determined using the Guinier approximation was 34.8 ± 0.7 Å (inset of Fig. 31). The distance distribution function, P(r), evaluated with GNOM program,[92] estimated the Rg and the maximum dimension (Dmax) of the molecule to be 35.30 ± 0.06 Å and 130 ± 5 Å, respectively (Fig. 32A).

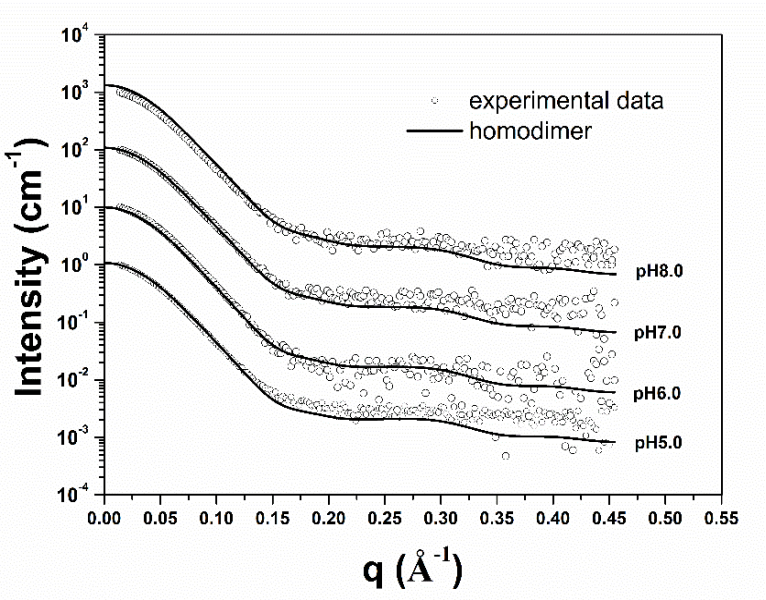

Figure 30 - Intensity (cm-1) versus q (A-1) data for BaFFase at 25 °C for pHs 5, 6, 7 and 8.

Source: By the author.

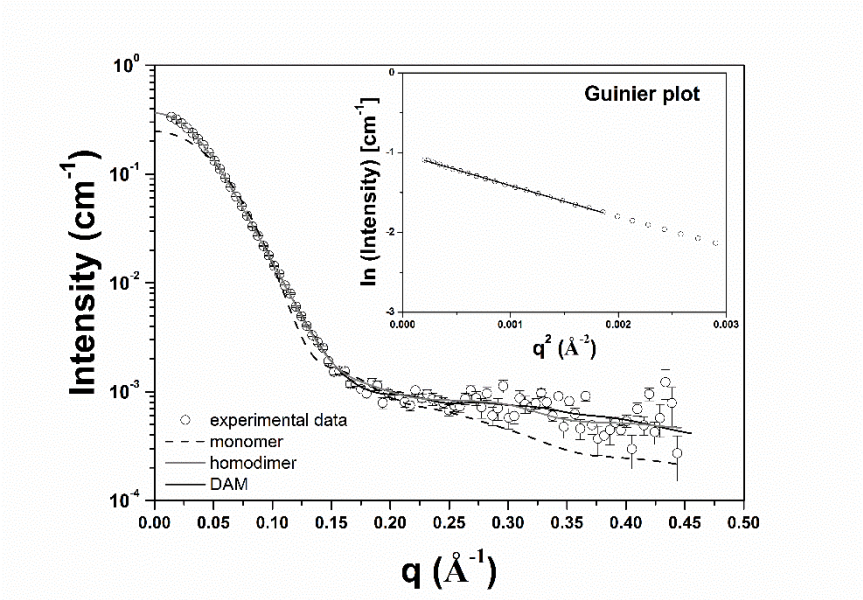

Figure 31 - Intensity (cm-1) versus q (A-1) plus the Guinier plot (ln I versus q2).

Source: By the author.

The GH32 crystallographic monomer has Rg = 27.03 Å and Dmax = 84.23 Å (Table 4). Thus, the radii of gyration determined from both the Guinier region of the X-ray scattering curve and that obtained with GNOM program are consistent with a homodimeric molecule for BaFFase in solution. Furthermore, the dimensionless Kratky plot (Fig. 32B) showed a curve with maximum value greater than 1.1 for qRg > $\sqrt{3}$, and that decay close to zero at higher qRg values, behavior consistent with a less globular protein displaying negligible flexibility in solution.[93]

Considering the results described above, we performed a search for biological assemblies in the crystallographic monomer structure of BaFFase employing the PISA server.[75] The analysis showed only one homodimeric biological assembly (Fig. 32), and the values determined for the buried solvent-accessible surface, free energy barrier of assembly dissociation and solvation free energy gain upon formation of the assembly were 3,490 Å2, 10.3 kcal/mol and -5.7 kcal/mol, respectively. The positive value determined for the free energy barrier of assembly dissociation indicates that the homodimer is thermodynamically stable. Figures 30 and 31 shows the experimental X-ray scattering curves for BaFFase measured at different pH values superimposed on the theoretical scattering curve based on the homodimeric assembly detected by the PISA server.[75] The fits are excellent and broadly consistent with a homodimeric molecule for BaFFase in solution. Furthermore, the

homodimeric assembly shows a molecule with Rg = 34.90 Å and Dmax = 135.5 Å, values that are in an excellent agreement with results obtained by SAXS analysis (Table 4).

The molecular shape of the BaFFase homodimer was determined from X-ray scattering curve with DAMMIN program.[72] The resulting consensus molecular envelope ($\chi 2$ = 2.3) obtained by SAXS is shown in figure 32. The molecular shape showed an elongated molecule, with a Dmax of approximately 130.2 Å and Rg = 35.20 Å (Table 5). Superposition of the molecular envelope and homodimeric structure detected by the PISA server showed excellent agreement and, therefore, consistent with a homodimeric molecule in solution.

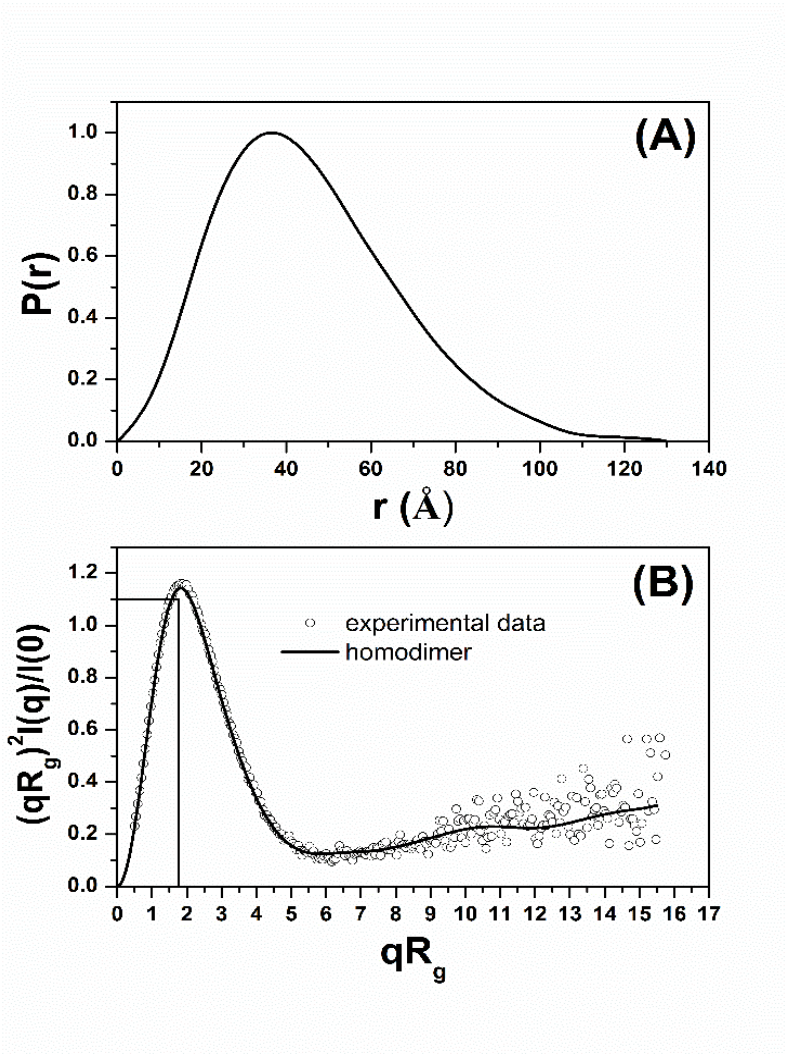

Figure 32 - (A) Experimental curve in function of distance distribution and (B) Kratky graph for data from BaFFase SAXS. Experimental data is shown in hole circles and the homodimer as a solid line.

Source: By the author.

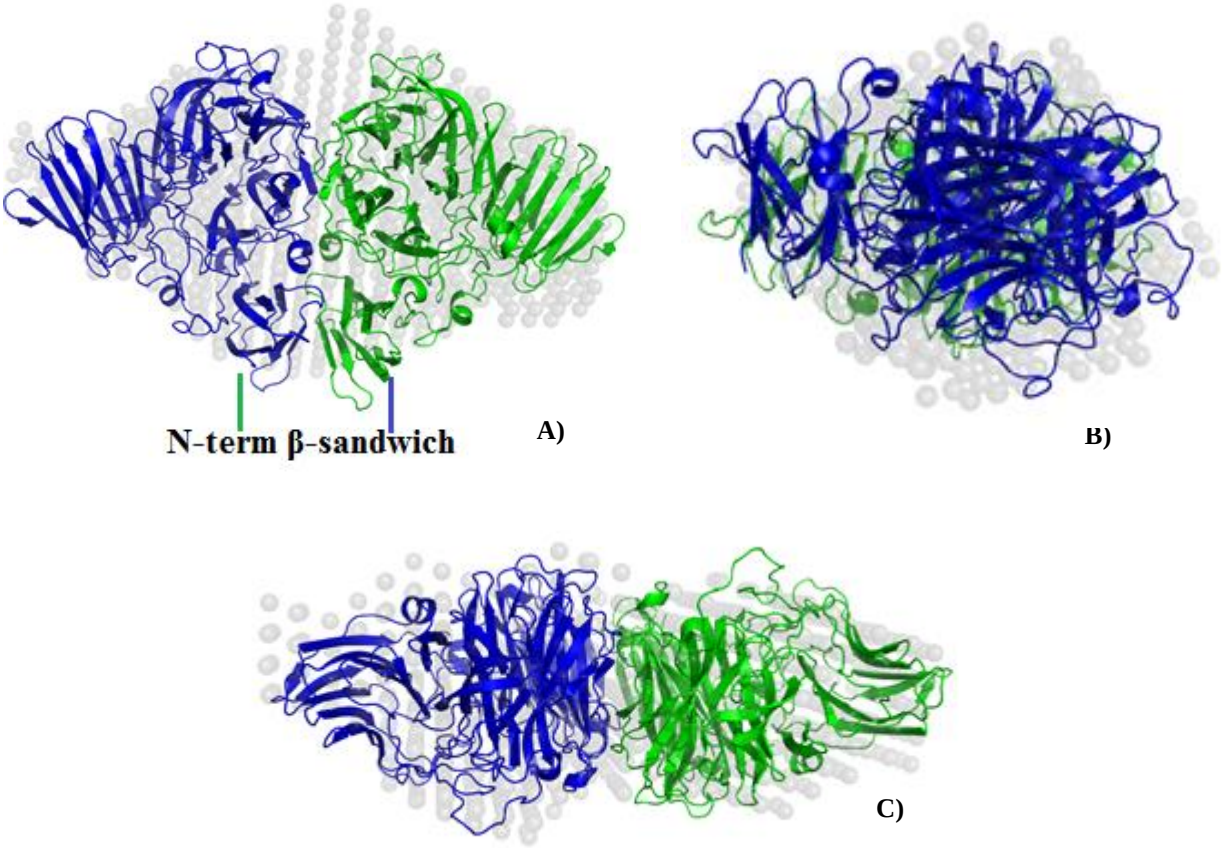

Figure 33 - Low-resolution model of BaFFase homodimer. Different views of the homodimer where the N-terminal β-sandwich domain is highlighted because increases the surface of contact within the dimer.

Source: By the author

Table 4 - General SAXS results.

	Rg (Å)	**Dmax (Å)**	**Res (Å)**	**χ2**
Experimental pH 8.0	33.9 ± 1.2 (1 mg/mL) 35.1 ± 1.5 (5 mg/mL) (Guinier) 34.86 ± 0.06 (GNOM)	130 ± 5	16	-
Experimental pH 7.0	35.1 ± 0.2 (1 mg/mL) 34.8 ± 0.7 (5 mg/mL) (Guinier) 35.30 ± 0.06 (GNOM)	130 ± 5	16	-
Experimental pH 6.0	36.1 ± 1.5 (1 mg/mL) 34.5 ± 0.5 (5 mg/mL) (Guinier) 34.71 ± 0.08 (GNOM)	130 ± 5	16	-
Experimental pH 5.0	34.9 ± 0.2 (1 mg/mL) 36.1 ± 0.8 (5 mg/mL) (Guinier) 36.11 ± 0.06 (GNOM)	130 ± 5	16	-
DAM pH 7.0	35.20	130.2	-	2.3
Monomer	27.03	84.23	-	-
Homodimer	34.90	135.5	-	1.9

Source: By the author.

5.3.10 Discussion of structural features

Following the reasoning from Bujacz, 2011. We can say BaFFase acts by a retaining mechanism. First, a nucleophile attack is performed by the carboxylate of the Asp 131 acting a primary nucleophile, forming the fructose-enzyme intermediate. The deduced fructose, remaining in the active site of the complex structure obtained by raffinose soaking, proves that the enzymes act with the overall retention of configuration on the anomeric carbon of fructose.

The β-strand 1, from blade I, is the more conserved region when compared with other β-fructofuranosidases (figure 23A). This region presents the RDPNG sequence, which lead to the conserved aminoacid Asp, which carboxylate group acts as a base donating a pair of electrons to the anomeric carbon of the fructose.

The nucleophile Asp (D-131) is located between the Asn 130 and the Pro 132 and belong to the conserved region **NDP**; Glu (E-375) is implicated in the acid base catalyst, these results corroborate what have been discovered for other GH32 members.[42,45,50,94] Asp 269 from the region **RDP** acts as the stabilizer of the transition state in the fructosyl transferring activity, the same results were found for a levansucrase from *Bacillus subtilis*.[91] The nucleophile Asp (D-131) is located at the first β-strand of blades 1 and 4 in the N-terminal β-propeller domain, which is exactly the same location of the catalytic residues from a β-fructofuranosidase from *B. longum* KN20.1[50] Asp (D-269) from the RDP sequence motif establishes two polar contact with oxygen 2 and 3 from fructose.

In the last decade GH32 enzymes from bacteria,[50,95] fungi,[67,96] and plants[45,65,97] have been intensively studied because of their well recognized role in biotechnology, but only monomeric enzyme were found. Surprisingly, studies on *Schwanniomyces occidentalis* fructofuranosidase (SoFfase)[98] and *Saccharomyces cerevisiae* invertase (ScINV)[42] showed that these enzymes from yeast were unique in that they form dimers mediated by their beta-sandwich.[54] In this research, we have proved that BaFFase, a GH32 member, is a stable homodimer in solution at 99.9%, even at various pH, thereafter, to our knowledge, this is the first oligomer crystal structure of GH32 enzyme belonging to the bacteria kingdom.

Additionally, we are describing a novel beta-sandwich domain not described before for a GH32 enzyme. Until now, only two domains were described for enzymes belonging to this Glycosyl hydrolase family (The N-terminal beta-propeller and the C-term beta-

sandwich). However, here we have proved that a GH32 member, BaFFase, also presents an additional beta-sandwich in the N-terminal domain, apart from the classical beta-propeller and the C-term beta-sandwich. This additional domain let the formation of the very stable homodimer described before. Taking on count that various authors suggest that the enzyme

6 Conclusions and perspectives

We studied an enzyme called a beta-fructofuranosidase from bacteria *Bifidobacterium adolescentis* ATCC 15703 which is a bacteria that promotes human heath by degrading indigestible saccharides for humans. We cloned the codifying region from this enzyme and expressed in *E. coli* Rosetta, then we studied its biochemical and structural features.

We found an enzyme with tolerant high temperatures, still 50°C, and especially tolerant to basic pH, maintaining activity of around 70% up to pH of 11.0. This enzyme, on contrary to other beta-fructofuranosidases from the same gender, is not affected by the presence on Zn2+ ion.

With respect to the structural features, we found that BaFFase present an additional β-sandwich in the N-terminal apart from the typical β-propeller and the C-terminal β-sandwich that other GH32 members share. Thereafter, in this research we are reporting the first oligomer crystal structure of a GH32 enzyme belonging to the bacteria kingdom. Additionally, we are reporting for the first time the presence of the N-terminal β-sandwich domain for an enzyme belonging to the Glycoside Hydrolase 32. And also we have seen that this additional domain increased the region of contact, thus letting the formation of the homodimer. However, to reveal with more exactness the function of the N-terminal β-sandwich, future experiments could be done where the region corresponding to this domain could be cut, and evaluate if this affect the oligomerization of BaFFase and if the enzyme remain active.

We have proved that not only fungi, but also bacteria GH32 enzymes can form oligomers. We think that our research directly increases the structural knowledge of GH32 enzymes, about the biochemistry of carbohydrates and the role of enzymes from Bifidobacteria genus.

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