CHARACTERIZATION OF SECRETED ACID PHOSPHATASE ACTIVITY OF A *STREPTOMYCES ALBUS* CLINICAL ISOLATE

By

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Abstract

Due to limitations in the efficacy of the current vaccines against Tuberculosis, there are efforts currently underway exploring the possibility of using the phylogenetically related Streptomycetes as heterologous vaccine vehicles against Tuberculosis. Ideal candidates for such a vaccine should be somewhat pathogenic and physiologically similar to *M. tuberculosis* so as to induce an immune response capable of targeting and inactivating this pathogen. Towards establishing such a physiological relatedness, previous work in our laboratory has established a similarity profile between *M. tuberculosis* and a clinical isolate of *Streptomyces albus* at the level of secreted enzymes. Amongst these was a strong secreted acid phosphatase activity. This work reports on the characterization of the secreted acid phosphatase activity in this clinical isolate of *Streptomyces albus*. In this work, two enzymes with sequence homology to those encoding a protein tyrosine phosphatase and an inorganic pyrophosphatase were purified from the culture supernatant of *S. albus*. The calculated molecular masses of these two putative phosphatases were approximately 18 and 30 kDa, respectively. The *S. albus* protein tyrosine phosphatase has 50% amino acid sequence identity to the protein tyrosine phosphatase of *M. tuberculosis* (gi|686037535). The inorganic pyrophosphatase has 68% amino acid sequence identity with *M. tuberculosis* inorganic pyrophosphatase (gi|625006479). Based on our knowledge of the role of acid phosphatases in pathogenic bacteria, presence of this activity in the supernatant of *S. albus* further supports the physiological relatedness of *M. tuberculosis* and *S. albus* and supports the proposition of using *S. albus* as a heterologous vaccine against Tuberculosis.

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List of Abbreviation

M	Molar
Mmol	Millimoles
µL	Microliter
µg	Microgram
mM	Millimolar
Abs	Absorbance
EDTA	Ethylenediaminetetracetic acid
h	Hours
kDa	Kilo Dalton
L	Liter
mg	Milligram
min	Min
mL	Milliliters
MW	Molecular weight
nmol	Nanomole
PAGE	Polyacrylamide gel electrophoresis
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
BCG	The live bacille Calmette-Guérin
TB	Tuberculosis
MTB	*Mycobacterium Tuberculosis*

S. albus	*Streptomyces albus*
Streptomyces SP	Streptomyces species
YM	Yeast Malt
TSB	Tryptic Soy Broth
Tris	Tris (hydroxymethyl) aminomethane
TEMED	Tetramethylethylenediamine
pNPP	p-Nitrophenyl Phosphate
rpm	Revolutions per minute
PI3P	Phosphatidylinositol 3-phosphate
OD	Optical density
NSAP	Bacterial non-specific acid phosphatase
NCBI	National Centre for Biotechnology Information
MALDI-TOF MS	Matrix assisted laser desorption ionization-time of flight
v/v	volume per volume
m/v	Mass per volume
V	Volume
TMBZ	3,3,5,5-tetramethylbenzidine
MUP	4-methylumbelliferyl phosphate
APS	Ammonium persulfate
Pi	inorganic phosphate

CFP	Culture filter protein
GBC	Fast Garnet GBC
PTPase	Protein Tyrosine phosphatase
FPLC	Fast protein liquid chromatography

1. Literature Review

1.1 Streptomyces

Streptomyces are members of the extensively disseminated Actinomycetes family. These bacteria are Gram positive in nature and in culture medium they replicate within 3 to 9 days (Lodders and Kämpfer, 2007). These microorganisms are quite significant, because presently they are used in making more than two thirds of all the naturally produced antibiotics being used on human and animals and also in many other pharmaceutical products including immune-depressants and anti-tumor agents. These bacterial produced products are synthesized by complicated secondary metabolic pathways (Bentley et al., 2002). The filamentous bacteria *Streptomyces albus* is a vital member of the family, and there is sufficient information about its genetic modifications (Zhou et al., 2004). This bacterium produces high quantity of extracellular proteins and is extensively used in different processes including expression, cloning and manufacturing of proteins that are heterologous in nature (Binnie et al., 1997).

The genome of *S. albus* has been completely sequenced. It has also been compared with other genomes that have been sequenced completely, for example *S. bingchenggensis* and *S. coelicolor* (Zaburannyi et al., 2014). The genomic sequence can be a good point to start better optimization of *S. albus* for using as a host in biotechnological processes for heterologous production of natural yields (Zaburannyi et al., 2014). Quick growth of the strain and its ability to sporulate in liquid culture are beneficial characteristics of *S. albus* in the laboratory and makes it a good model bacterium for experiments related to heterologous expressions. It has also been used as a model to study the basic biological processes of actinobacteria, like morphogenesis, growth, formation of cell wall, cell division and resistance to antibiotic (Zaburannyi et al., 2014).

For long *S. albus* has been used in heterologous production of different secondary metabolites starting from secondary metabolites of Micromonospora (Lombo et al., 2006) to potential anti-cancer agents (Baltz, 2010). To put examples, *S. albus* was employed for expressing steffimycin biosynthetic genes (Gullón et al., 2006), and many other including, isomigrastatin (Feng et al., 2009), thiocoraline (Lombo et al., 2006), fredericamycin (Wendt-Pienkowski et al., 2005), cyclooctatin (Kim et al, 2009), napyradiomycin (Winter et al., 2007), and biosynthetic moenomycin (Makitrynskyy et al., 2010) gene clusters. The total sequence of the genome focuses on its minimized volume along with new dimensions for application of *S. albus* (Zaburannyi et al., 2014).

1.2 Streptomyces – Pathogenicity

Only a very minimum number of strains from the Streptomycetaceae family show pathogenic effects on plants and animals including humans. Although Streptomycetes are more known for their benefits, like the production of antibiotics, pathogenic Streptomycetes should not be ignored. One pathogenic species from the family is *Streptomyces somaliensis*. This is a human pathogen and is responsible for actinomycetoma, which is a local skin infection which with time affects the tissues and bones underlying the skin. This infection might result into deformation of body parts and even total amputation of the effected body part (Lodders and Kämpfer, 2007). The condition is common in arid tropical and subtropical areas. Cases of Actinomycetoma have been reported from many African nations such as Somalia, Mali, Senegal and Niger. The disease has also affected North and South American nations such as Argentina, Mexico and Venezuela. These microorganisms live in the soil, and they get into the body of human most commonly through the feet. This condition can be efficiently treated with proper antibiotics, but in poor nations, where proper medical facilities are not available this disease can have a devastating impact. Another condition caused by strains of

Streptomycetes is farmer's lung disease. This is an allergic reaction caused by inhalation of the bacteria living in the hay dust or dust from other agricultural products. However, this condition can also be caused by other microorganisms apart from Streptomycetes. Exposure to bacteria strains generated in the molded hay or forage can cause lung infections such as pneumonia or bronchitis (Lodders & Kampfer, 2007).

Streptomycetes is a part of the microbiota of the soil, and can work as a severe pathogen for plants. It attacks the underground plant parts, the roots, destroying the plant and can result in economical loss due to spoiling of the roots of turnips, radishes or potatoes. The most severe plant pathogen from the family is *Streptomyces scabies*, which causes potato scab, a deformation of the skin of the potato and its actions are not limited only to potatoes (Lodders & Kampfer, 2007).

1.3 Pulmonary infection by *Streptomyces albus*

The bacteria genus Streptomyces is a widely spread and commonly occurring microbe in the environment but it has a low rate of severe invasion infections. Through our literature review we were able to point only 21 incidents of Streptomyces infections, in which 8 were pulmonary. For each of the case there was also a contributing factor. Examples include: immunosuppression related to infection of HIV (Dunne et al.., 1998), use of oral (Kapadia et al.., 2007) or inhaled corticosteroids (Kofteridis et al.., 2007), Crohn disease (Ekkelenkampea et al.., 2004), occurrence of a foreign particle like a central venous catheter (Carey et al.., 2001), antineoplastic chemotherapy (Moss et al.., 2003) or a prosthetic aortic valve (Mossad et al.., 1995). Death in these cases were mostly linked with the underlying disease, and it had not been associated to the infection of Streptomyces. Two possibilities were explored to find out how the patients got the Streptomyces infection. Firstly, immune-deficiency is induced by sarcoidosis (Iannuzzi et al.., 2007), which is clinically used after subcutaneous injections to

energize tuberculin or the other immunogenic haptens. Growth of controlling T lymphocytes (Iannuzzi et al.., 2007) and attenuated-myeloid dendritic cell actions (Mathew et al.., 2008) reduces the efficiency of cellular immunity and enhances chance of infection in patients already affected. Secondly, vulnerability to infections can be increased due to splenectomy. These infections majorly include bloodstream infection by capsulated bacteria or unscrupulous infection by *Pneumocystis jiroveci, Campylobacter jejuni* or *Babesia* spp (Mathew et al., 2008). The Streptomyces lung infection of the patient was not attained through bloodstream, rather by direct contact with the air. At the same time, other immune mechanisms that are not linked with blood, like scarcity of particular lymphocyte populations or dysregulation cannot be ruled out. Presence of granulomas, with or without focal necrosis is another pathogenic characteristic of Streptomyces infection to pulmonary organs (Iannuzzi et al.., 2007). These make it really difficult to differentiate the infections caused by these organisms from tuberculosis, and hence to ensure the diagnosis, bacterial culture is often used. The histologic difference amongst the 2 units is not specifically defined because invasive infection by Streptomyces is quite rare. In case of the patient under observation, granulomas related to sarcoidosis and a non-particular, amorphous eosinophil based substance, which was not caseous necrosis, was revealed by the histological investigations. Both the injuries might also have been caused due to Streptomyces infection (Figure 1). To characterize even further, it was suggested by (Dunne et al, 1998) to add sulfur granules to the particular histological sample of Streptomyces infection (Dunne et al., 1998). A thorough literature review on the results of Streptomyces in vitro testing pointed out a general vulnerability to macrolides, aminoglycosides, sulfamethoxazole / trimethoprim or imipenem. As per this finding, the first line of treatment

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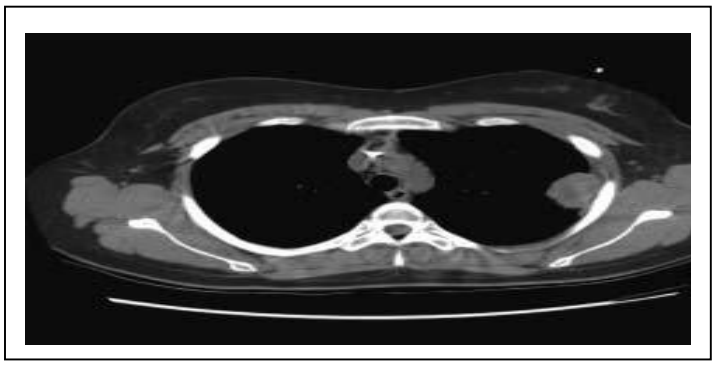

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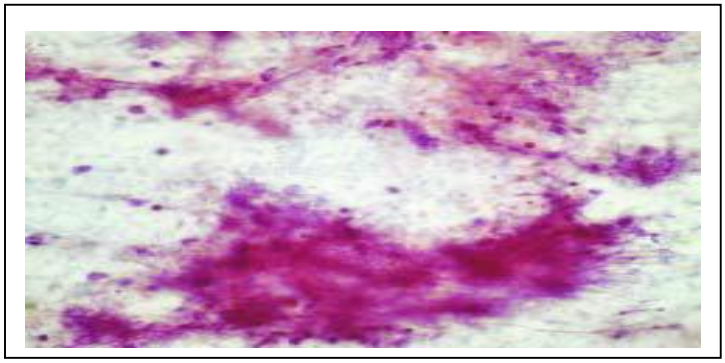

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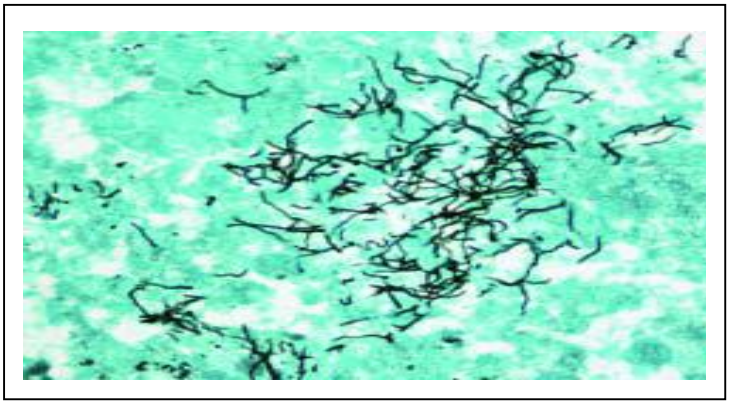

Figure 1. Lung nodule shown on chest infected by *Streptomyces albus*. **(A)** computed tomography (B) A fine-needle aspirate of the lesion stained with Papanicolaou, ×400. (**C)** Gomori methenamine silver stain, ×1000). Reproduced with permission from Kapadia et al., (2007).

in case of invasive Streptomyces infections, should start with aminoglycosides and imipenem at least for the first six weeks. In inference, it is to be noted, that invasive *Streptomyces* caused lung infections should be taken under differential diagnosis in case of immunocompromised patients with interstitial pneumonia (Riviere et al., 2012).

1.4 *Streptomyces* and *Mycobacterium* --- Similarities

Both Streptomyces and Mycobacterium belong to the phylum Actinobacteria, and show no similarity at the first sight. The microorganisms under *Streptomyces spp*. form spores and have a filamentous body. On the other hand, the bacteria of Mycobacterium species are non-sporulating and bear a rod like shape. However, the latest genetic and cell biology based studies have found similarities in the developmental as well as morphological features of the two species. Realizing the processes behind these similarities and the difference in development and morphogenesis of these two bacteria species aids in understanding the evolution of shapes in Actinobacteria and on the other will also give vital information about better medical treatments (Scherr and Nguyen, 2009). Comparing the genome of *S. coelicolor* with the genomes of the two bacteria, *M. bovis* and *M. tuberculosis* that belong from Actinomycetes, high similarity was found at the level of individual gene sequence. It was found that 761 (19.42%) and 740 (18.56%) proteins in *M. bovis* and *M. tuberculosis* respectively, bear more than 50% identity with the proteins of *S. coelicolor*. In these genes there were many open reading frames which encode membrane proteins and also different hypothetical proteins without any particular function. Global comparison (Figure 2) also pointed out a conserved gene arrangement amongst the total genome of the mycobacteria and that of *S. coelicolor* (Arzuaga et al., 2011).

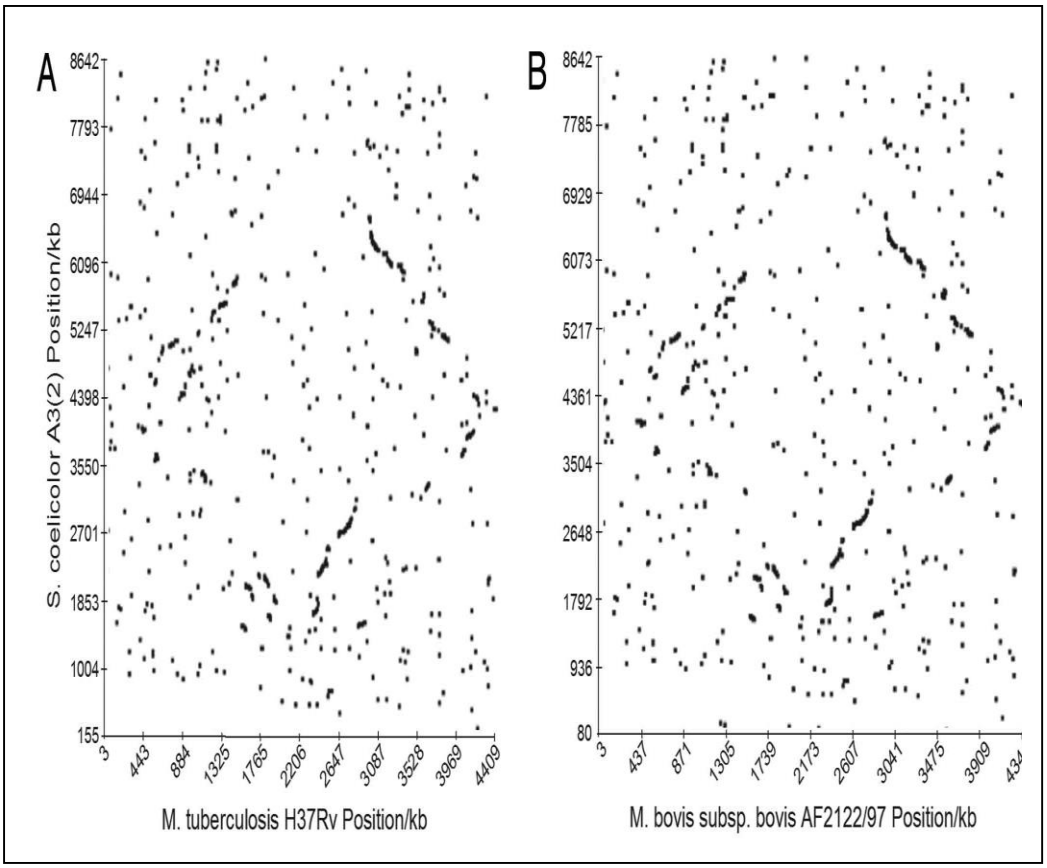

Figure 2. Protein scatter plot results. (A) Total protein hits for *M. tuberculosis* H37Rv vs. *S. coelicolor* A3. (B) Total protein hits for *M. bovis* AF2122/97 vs. *S. coelicolor* A3. Protein hits were determinate from blast searches. Dots indicate two genes that a similar (a match between *M. tuberculosis* or *M. bovis* and *S. coelicolor*) one to another. Axes represent the positions of the proteins in the genome in the order in which they occur on the chromosomes. Reproduced with permission from Arzuaga et al., (2011).

It has been demonstrate that the immunogenic capacity of Streptomyces offered protection to an extent against the mycobacterial murine infection model with *Mycobacterium bovis* Bacille Calmette-Guérin (BCG). These findings together with the fact that Streptomyces bears the potentiality for expression of foreign proteins makes this bacterium a perfect host to be used in designing vaccines for tuberculosis (Arzuaga et al., 2011).The two genera Mycobacterium and Streptomyces are probable to bear more similarities than expected. Even after the unique ways they impact the life of humans, their different life cycles and shapes, these two bacterial species from the Actinobacteria family bear similar modes of growth and developmental patterns (Arzuaga et al., 2011).

1.5 Nonspecific secreted acid phosphohydrolases (NSAPs)

Different bacteria are capable of secreting different enzymes that can cause de-phosphorylation of different organic compounds; and these enzymes have different vital or accessory parts in the physiology of bacterial cells. Most of the de-phosphorylation reactions that take place in the prokaryotic cells either include phosphoester hydrolysis or hydrolysis of the phosphoanhydride bonds; and these reactions are catalyzed by a collection of enzymes known as phosphatases or phosphohydrolases (Boyer et al, 1961). Secretion of some of these enzymes occurs outside the cell membrane, and they are either secreted in a soluble form or they are kept as membrane-bound proteins. This group of enzymes, which are being referred to as secreted phosphohydrolases are thought to work on organic phosphoester, like, sugar phosphates, nucleotides and phytic acid scavengers which are prohibited from entering the cell membrane. The organic by-products and the Inorganic phosphate Pi released might be transported into the cell through the membrane and in this way the cell gets its essential nutrient molecules (Wanner, 1996). Some of the secreted phosphohydrolases have also evolved for serving specific functions that are linked with microbial virulence. For example, acid

phosphatases of *Legionella micdadei* (Dowling et al, 1992) which inhibits respiratory burst and *Francisella tularensis (*Reilly et al, 1996), *Salmonella enterica ser. typhimurium* (Kaniga, 1996), and *Yersinia spp*. (Guan and Dixon, 1990) Different other phosphohydrolases remain present in the cytoplasm, where they are involved in dephosphorylation reactions taking place for signal transduction and also in many metabolic pathways.

NSAPs or bacterial nonspecific acid phosphohydrolases are enzymes that are secreted as periplasmic proteins that are soluble or as lipo-proteins that are bound with the membrane; these are usually capable of de-phosphorylating a wide range of structurally distinct substrates and shows accurate catalytic actions at neutral to acidic pH. The NSAPs of bacterial origin are either monomeric or oligomeric in nature that contains polypeptide constituents with a 25–30 kDa *M*r. Depending on the link present in the amino acid sequence, 3 separate families of NSAPs have been identified. These are termed as molecular class A, B and C respectively. The NSAPs that belong to the same class bear some common features in the functions and biophysical properties; but might show differences in functionality. NSAPs have been identified in different taxa of microbes and the same species of bacteria can produce enzymes of different classes. Within the different NSAPs of bacterial origin phyletic as well as structural links can be observed; the same can be found for eukaryotic phosphohydrolases. The present information available on bacterial NSAPs is being reviewed in light of the analytical instruments which might be effective to characterize them. An overview on the use of the NSAPs of bacterial origin in biotechnology has also been presented (Rossolini et al., 1998).

The present knowledge we hold about NSAPs of bacterial origin can be summarized as: (i) NSAPs of bacterial origin are widely spread enzymes present in many taxas of microorganisms; (ii) all the NSAPs that have been isolated and identified so far from different bacteria are secreted enzymes, some of which lipoproteins bound to the membrane and some

are secreted as periplasmic proteins that are soluble in nature; (iii) Depending on their similarities at the sequence level, a minimum of 3 different molecular families of bacterial NSAPs have been observed; and the constituents of these classes have been demarked as Class A, B and C; the signature sequence motifs particular to each of the molecular class have been characterized which can be applied to identify novel hypothetical proteins; in addition with the similarities between sequences, the constituents of each of the molecular classes bear some general biophysical and functional features that can be used as phenotypic markers for probable characterization of the newly revealed enzymes; (iv) even after the existing similar features, contributors of each of the NSAP molecular classes show difference in function pointing out that, amongst a molecular class, different functional enzymes have evolved; as a matter of fact, though a large number of NSAPs are active against a wide range of substrate, some demonstrate a narrow range in substrate selection; (v) NSAPs of more than one molecular families can be present within the same species of bacteria, and here enzymes of different class plays different physiological roles; (vi) The conserved structural motifs are shared within the different NSAPs of bacterial origin and some of the other eukaryotic and bacterial phosphohydrolases, making the enzymes an interesting point for molecular evolution researches and comparative enzymology (Rossolini et al., 1998).

The molecular class A acid phosphatases are defined as a collection of phosphohydrolases secreted by bacteria; these phosphohydrolases include a polypeptide component along with an *M*r ranging from 25-27 kDa and exhibit conserved sequence motifs. The products of 6 different class A phosphatase encoding genes have been typified to different extents through cloning and sequencing. Presence of different conserved domains for the enzyme family was found by comparison of the amino acid sequences of the 6 class A enzymes and a signature sequence motif has been described as G-S-Y-P-S-G-H-T. A set of secreted phosphohydrolases of bacterial origin are included in the molecular class B acid phosphatases;

these secreted phosphohydrolases includes a polypeptide element which has a *M*r of around 25 kDa and conserved sequence motifs that are shared. The size of the polypeptide is quite similar to the NSAPs belonging from class A, but they have noteworthy differences at the level of sequence. The presence of different highly conserved domains can be observed through amino acid sequence comparison. For the bacterial Class B NSAPs the motif D-I-D-D-T-V-L-F-S-S-P might be suggested as a signature pattern of sequence. The NSAPs of molecular class B, also appear to be remotely related to the NSAPs of class C, at the level of sequence; the similarity is also observed in case of some of the plant acid phosphatases. The molecular class C phosphatases have just been revealed as a collection of secreted lipoprotein of bacterial origin possessing NSAP actions; these phosphatases includes a polypeptide constituent which has around 30 kDa *M*r and shares motifs of conserved sequences. The class C enzymes appear to be remotely related with that of class B enzyme NSAPs and some plant acid phosphohydrolases at the sequence level. Conserved sequence motifs between the class C enzymes, and some other eukaryotic or bacterial proteins, including the Class B NSAPs and some acid phosphatases of plant origin were noticed through comparison with other sequenced proteins. Depending on these revelations we can conclude that the bacterial NSAPs of molecular class A and B, along with their homologues of plant origin, are members of the same phosphohydrolases superfamily. In the most conserved domains four variants of aspartate residues were observed, which led to the superfamily as "DDDD". These enzymes are expected to be related phylogenetically as well as mechanistically; and the most conserved motifs are probably vital for functioning of the enzyme and can even have a role in the catalytic cycle (Rossolini et al., 1998).

1.6 SapM or Secreted acid phosphatase of *Mycobacterium tuberculosis* and its role in pathogenicity:

Though our knowledge on *Mycobacterium tuberculosis* has increased rapidly, success in controlling TB has not been able to meet satisfactory results, which is evident from the fresh cases of tuberculosis that occur every year across the world which number in the millions (WHO, 2012). The situation has become even more serious with the emergence of the pathogenic strains that are highly drug resistant (XDR) and multidrug resistant (MDR) and occurring with increasing number of co-infections, like HIV-TB. There are 1.1 million new cases of TB-HIV co-infection, 25,000 new cases of XDR-TB infections and 440,000 new cases of MDR-TB infection reported globally, every year (WHO, 2011). *M. tuberculosis* ability to cause disease rests resides in the bacterium's ability to inhibit the host immune reaction. *M. tuberculosis* pathogen enters the body with inhalation of aerosolized droplets from an infected person. Once the bacterium is inhaled it makes its way to the alveoli of the lungs where macrophages engulf the bacteria (Li and Xie, 2011). The impact of the infection majorly rest on the dealings between the pathogen and the host, mostly within the macrophages (Chastellier, 2009). The defense offered by alveolar macrophages is curbed by *M. tuberculosis* in many ways to continue its survival. The inhibition of phagosomal maturation is considered as one of the key strategies used by the pathogen (Chastellier, 2009).

M. tuberculosis Rv3310 includes an acid phosphatase known as SapM (Saleh and Belisle, 2000), which shows features of a potential virulence factor in the *M. tuberculosis* pathogenesis. Firstly, SapM is a secreted functional protein of 28 kDa and contains a signal peptide sequence typical of prokaryotes, which was observed to be present in the extracellular fraction. The hypothetical protein of Rv3310 includes 299 amino acids. Secondly, the enzyme has noteworthy action in the pH range 5.5-6.8 with optimal activity at pH 6. This pH range

which is optimal for this enzyme activity shows similarity with the optimal pH observed for phagosomes containing mycobacteria and non- specific acid phosphatase of bacterial origin. In addition to that, the SapM amino acid sequence does not include the signature motifs that define any of the three bacterial NSAP classes. Actually, a new class of NSAPs of bacterial origin that might work as a histidine phosphatase can be observed in SapM (Saleh and Belisle, 2000). Presence of PI3P (phosphatidylinositol 3-phosphate) on phagosomes is vital for the phagolysosomal fusion (Vieira et al, 2002). This lipid constituent takes part in the Rab effector proteins, such as, tyrosine kinase substrate (Hrs) that is regulated by hepatocyte growth factor and EEA1(early endosomal autoantigen 1) docking; which is indispensable for maturation of phagosomes (Vieira et al,2002; Fratti et al, 2001; Vieira et al, 2001). SapM is a secreted phosphatase which dephosphorylates PI3P (Vergne et al, 2005; Saleh and Belisle, 2000). However, different observations have been reported through many studies about the contribution of SapM in inhibiting maturation of phagosomes. According to a study performed on BCG, phagosomes that dock on the killed BCG insistently carry PI3P; but it is detached from the phagosomes docking on live BCG (Vergne et al, 2005). According to an in vitro fusion assay study performed with the *M.tuberculosis* produced SapM protein, which hydrolyses PI3P, also suggested that, in vitro, SapM arrests fusion of phagosome-late endosome. These observations suggest the involvement of SapM in disrupting the maturation of phagosome in *M. tuberculosis* infection (Figure 3). However, as per the studies performed on phagosomal maturation in THP-1 macrophage infection with wild strain and its sapM mutant (DsapM), fbpA mutant (DfbpA) and also double knock-out mutant (DsapMDfbpA), it has been observed that the DsapMDfbpA mutant was highly aligned with the lysosomal markers, and was followed by DsapM, DfbpA as well as the parental strain, which indicates the contribution of SapM in inhibiting the maturation of phagosomes (Saikolappan et al, 2012).

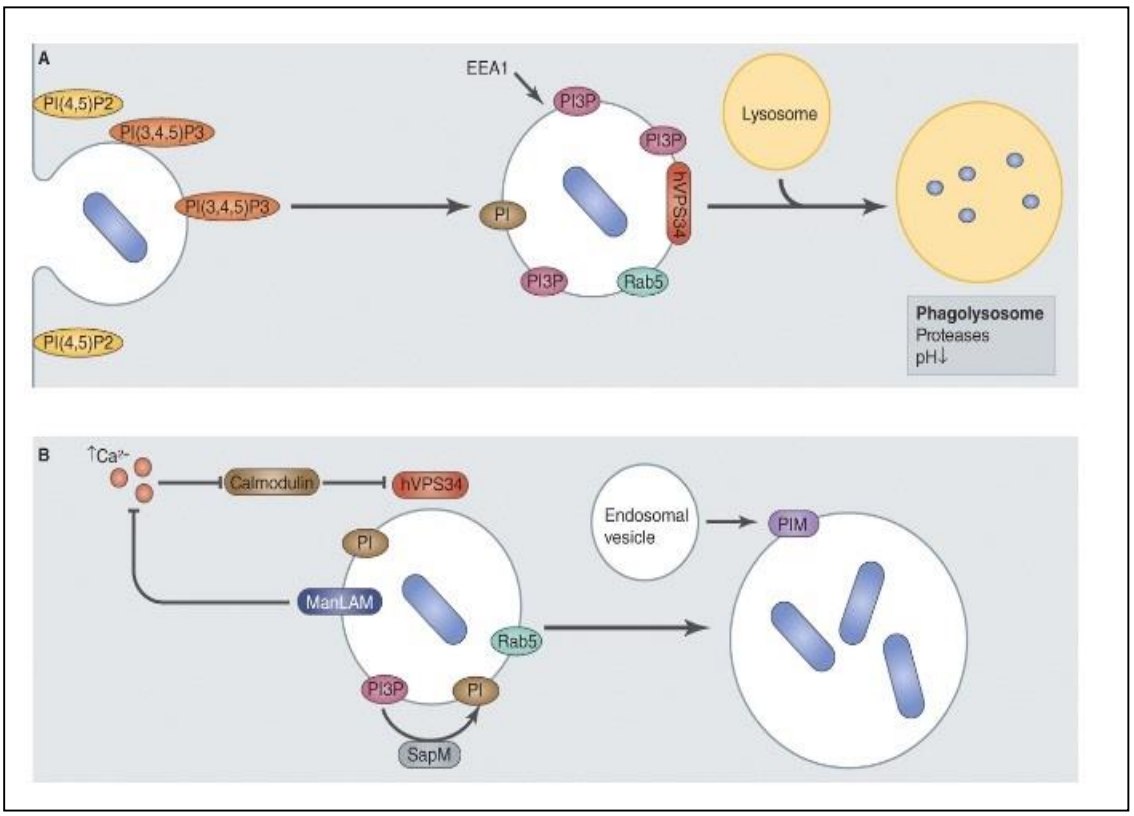

Figure 3. Intracellular pathogenesis and the phagosomal internalization and degradation of microbes. (A) Normal maturation of a phagosome containing nonpathogenic bacteria. After phagocytosis, bacteria reside in a vacuole showing similarities with early endosomes, microbes are internalized and the nascent phagosome fuses with early endosomes to form mature phagosomes that then fuse with late endosomes to become late phagosomes (LP). LPs fuse with lysosomes which results in the degradation of the microbe. (B) Attenuated phagosome maturation by the pathogen circumvents this process, allowing proliferation and even escape into the cytoplasm (Alix et al., 2011).

1.7 Research Rationale

Tuberculosis is responsible for nearly 1.4 million deaths globally every year and continues to remain a serious threat to human health. The problem is further complicated by the growing incidence of multidrug-resistant TB and extensively drug-resistant TB, emphasizing the need for the development of new drugs against this disease. The entry of *M. tuberculosis* into macrophages and subsequent events appear to involve specific signals between the host cell and the bacterium, suggesting that molecules may be necessary for the reprogramming of the host signaling network that helps the bacterium in its propagation. *M. tuberculosis* is known to secrete a large number of proteins into the extracellular medium. These secreted proteins play an important role in the interaction of mycobacteria with the host cell, and they are thought to be prime candidates for the development of subunit vaccines and new antimycobacterial drugs. Secretory acid phosphatase (SapM) of is known as a virulence factor and displays optimum enzyme activity at pH6, an acidity consistent with mycobacteria –containing phagosomes. SapM was identified by Saleh and Belisle (2000). Secretory acid phosphatase (SapM) of is known to dephosphorylate phosphotidylinositol 3-phosphate (PI3P) present on phagosomes (see Figure 3). However, there have been divergent reports on the involvement of SapM in phagosomal maturation arrest in mycobacteria, and there have also studied whether SapM is essential for the pathogenesis of. By deleting the sapM gene of, it has been demonstrated that MtbDsapM is defective in the arrest of phagosomal maturation as well as for mycobacterial growth in human macrophages. The importance of SapM in phagosomal maturation arrest as well as in the pathogenesis of establishes it as an attractive target for the development of new therapeutic molecules against tuberculosis (Puri et al, 2013). Not only in *M.tuberculosis*, but also in some bacteria, protein phosphorylation plays an important role in sensing extracellular signals and coordinating intracellular events. Thus, it is not surprising that in pathogenic bacteria, such as *Yersinia pseudotuberculosis, Salmonella enterica serovar Typhimurium,*

Francisella tularensis and *Legionella micdadei* phosphatases act as major virulence determinants. Recently, comparative genome analysis between Streptomyces and *Mycobacterium tuberculosis* suggest that both descend from a common Actinomycete ancestor and revealed much similarity at the level of individual gene sequences. They have over 50% of identity with *S. coelicolor* proteins. According to this similarity and since this an uncharacterized pathogenic *Streptomyces albus* isolate (whose secreted phosphatases has not been yet identified) from the lungs, in this study we hypothesize that it secretes specific virulence factors common with other respiratory pathogens. These would include acid phosphatases. The reason for choosing this pathogenic Streptomyces strain in this study (a clinical isolate *S. albus*): (1) because it recently found to be one of the streptomyces infections which causes pulmonary infections and pathogenic characteristic of Streptomyces infection to pulmonary organs (Presence of granulomas, with or without focal necrosis) make it really difficult to differentiate the infections caused by, and hence to ensure the diagnosis, bacterial culture is often used. The two species Mycobacterium and Streptomyces are probable to bear more similarities than expected. (2) It is known that live pathogenic bacteria are processed differently than nonpathogenic bacteria in macrophages, suggesting that live pathogenic bacteria are able to express specific virulence factors triggered by the environment of the phagosome, which interfere with host cell signaling and attenuate maturation of the phagosome.In support of this hypothesis, the result of the western blot assay that has been done in our lab by (Aljassim, 2015) seven monoclonal antibodies specific for seven distinct antigens of *M. tuberculosis* were used to screen for cross reactivity with the secretory fractions of *S. albus.* Of the seven antibodies, only one gave a positive reaction. This was a monoclonal antibody directed at a specific internal amino acid sequence in the secreted acid phosphatase of mycobacteria (SapM). The existence of a homologous secreted acid phosphatase of Streptomyces (SapS) further supports the physiological relatedness of *M. tuberculosis* and

Streptomyces albus and points to possible similarities in their pathogenesis. This makes the *Streptomyces albus* a prime candidate for the development of subunit vaccine against TB.

1.6 Research objectives:

The main research objective of this study is the purification and characterization of the secreted acid phosphatase of a clinical isolate from S*treptomyces albus*, the homolog of which is implicated in the virulence of *Mycobacterium tuberculosis* using biochemical assays and a proteomics approach. The specific objectives are:

(1) Follow the secretion pattern of the enzyme using zymography and colorimetric assays.

(2) Fractionate culture supernatant using FPLC and purify the secreted acid phosphatase.

(3) Identify the enzyme using tryptic digest and mass spectrometry and/or and N- terminal sequencing.

2. Experimental Methods

2.1 Chemicals

All chemicals, unless stated otherwise, were purchased from Sigma-Aldrich CO and were of the highest purity available. Ion exchange column were purchased from GE Healthcare. Protein electrophoresis reagents were obtained from Bio-Rad Laboratories. SDS-PAGE molecular weight standards were obtained from FroggaBio and Fisher Scientific.

2.2 Bacterial strain and culture conditions

Streptomyces albus strain NML no.05-0087 was obtained from the Public Health Agency of Canada on September 2011. To grow this strain , an inoculum was cultured on yeast malt (YM) agar (Bacto TM) containing per liter (Dried malt extract 3 g ; Yeast extract 3 g; Peptone 5 g; Dextrose 10 g and agar 20 g) for 3 days at 37°C . For liquid media, spores were inoculated in 50 mL Tryptic Soy Broth medium (Bacto TM) containing, per liter (Pancreatic digest of casein 17 g; Papaic digest of soybean meal 3 g; Sodium chloride 5 g; Di-basic potassium phosphate 2.5 g; Glucose 2.5 g; pH 7.2.) for 36 h and 25 mL of this culture was used to inoculate 500 mL Sauton's medium (containing, per liter: 0.5 g Potassium Phosphate KH_2PO; 0.5 g Magnesium Sulfate $MgSO_4$; 2.0 g Citric Acid Anhydrous Certificate A.C.S; 0.05 g Ferric Ammonium Citrate (Fisher Scientific) and 4.0 g Ammonium Chloride NH_4Cl; 48g Glucose pH 7.4) After 7 days growth in a rotary shaker (Inova 4000, New Brunswick Scientific) at 30°C and 200 rpm, the mycelium was removed by centrifugation at 8500xg (Sorval RT 6000D centrifuge) for 30 min and the supernatant was used for the purification of the enzymes.

To study the effect of phosphate ion concentration on acid phosphatase production and secretion, the bacteria were cultured in Sauton's medium containing three different concentrations of inorganic phosphate (KH_2PO), 0, 0.2 and 2 g/L. The extent of bacterial

growth was assessed by measuring the optical density of the cultures at 600 nm (OD600) using Shimadzu UV spectrophotometer (Hitachi, Tokyo, Japan) and the growth was monitored for 10 days. For studying the effect of nitrogen source on acid phosphatase, Asparagine in the original Sauton's was substituted with three different nitrogen sources, Ammonium chloride, Glycine and Sodium nitrate.

2.3 Preparation of supernatants and cell wall bound proteins

Culture supernatants (500 mL) were obtained by centrifugation followed by filtration (0.22 mm pore size) and in some cases were concentrated through P-3 membranes in an Amicon ultrafiltration system to a final volume of 5 mL without appreciable loss of activity or ammonium sulfate solution was added to the concentrated supernatant preparation (20 mL) and the protein precipitated in the range of 40-70% saturation. The protein pellet was collected by centrifugation and resuspended in 5 mL of 25 mM sodium acetate buffer pH 6. The final volume then dialyzed in a dialysis bag (molecular porous membrane tubing spectrum) against the same buffer at 4°C overnight with several changes (Moura et al, 2001).

Extraction of bacterial cell wall proteins with Triton X-100, growing bacterial cultures were harvested by centrifugation (6,000 × g for 10 min) and washed with sodium acetate buffer twice at room temperature. Pelleted bacteria were immediately resuspended in approximately 0.5% of the original culture volume, using buffer containing 2% (vol/vol) Triton X-100. Resuspended bacteria were incubated for 15 min at 37°C with gentle shaking, Bacterial suspensions were centrifuged at 15,000 × g for 10 min; the supernatants were aliquoted and stored at −20°C (Moura et al, 2001).

2.4 Protein quantitation

Protein concentrations were measured by the Bradford reagent (Bio-Rad) and bovine serum albumin was used as standard. The Bio-Rad protein assay is a colorimetric assay for measuring total protein concentration and is based on the Bradford dye-binding method (Bradford 1976). Comparison to a standard curve provides a relative measurement of protein concentration. Protein solutions were assayed in duplicate at 595 nm absorbance (MRX Microplate Reader) and the assay was performed according to the manufacturer's instructions supplied with the kit.

2.5 Acid phosphatase activity assay

Acid phosphatase activity was assayed by measuring the released *p*-nitrophenol at 405 nm at pH 6 from the hydrolysis of 5mM p-nitrophenylphosphate (*p*NPP). All assays were performed in a microtiter plates in a volume of 200 µl. Activity initiated by adding 20 µg/µl of enzyme solution to 100mM sodium acetate(MP Biomedical) buffer pH 6 containing 5 mM *p*-nitrophenyl phosphate (PNPP) and 1 mM $MgSO_4$ (Fisher Scientific) , and incubated at 37°C for 30 min. Since *p*-nitrophenol (*p*NP) is colorless at acid pH, concentrated 5N NaOH (50 µl) was added to the reactions after the appropriate incubation at 37°C and the absorbance of the *p*-nitrophenol formed was measured at 405 nm (MRX Microplate Reader). The determinations were performed in triplicates and a unit of enzyme activity was defined and expressed as the amount of enzyme that releases one nmol of *p*-nitrophenol (ε= 16,900 l/ mol.cm) per minute, per milligram of protein. Acid Phosphatase from wheat germ was used as a positive control and sterilized medium as a negative control (BISC 429). To measure alkaline phosphatase activity, the *p*NPP hydrolysis reactions were performed in 100 mM Tris (pH 10).

2.6 Polyacrylamide gel electrophoresis (PAGE)

2.6.1 SDS-PAGE

Denaturing SDS-PAGE was performed as described by LaemmLi (1970). Polyacrylamide gels containing SDS were made of a 12 % separating gel with a final volume of 5 mL (2.15 mL H_2O, 1.25 mL 1.5 M Tris-HCl pH 8.8, 1.5 mL 40% acrylamide / 0.8 % bis-acrylamide (w/v), 0.05 mL 10% SDS , 0.05mL 10% (w/v) ammonium persulfate (APS), and 2 µl tetramethylethylenediamine (TEMED).

5% stacking gel of total volume 2 mL containing (1.46mL H2O, 0.25 mL 0.5 M Tris-HCl pH 6.8, 0.02 mL 10% SDS, 0.25 mL 40% acrylamide / 0.8 % bis-acrylamide (w/v), 0.02 mL 10 % (w/v) APS, and 2 µl TEMED). Prepared samples were combined with 2X sample buffer (3.8 % SDS, 38% glycerol, 0.1 M Tris-HCl pH 6.8 and 0.02 % bromophenol blue) to a final concentration of 1X (LaemmLi, 1970). The gels were ran at a constant voltage of 200 V for 45 min in a Mini -PROTEAN Tetra System (BIO-RAD) with 1x SDS running buffer (144 g glycine, 1/10 dilution of 30 g Tris-HCl, 10 g SDS in 1 L dH2O; pH 8.3) . SDS-PAGE was carried out at room temperature and stained with either Silver nitrate or Coomassie brilliant blue R-250 stains.

2.6.2 Native-PAGE

Non-denaturing PAGE was carried out in the same system above but omitting SDS from the sample buffer, electrode buffer, and polyacrylamide gels. Native-PAGE was done at 4ºC to prevent loss of activity. β-mercaptoethanol and the boiling treatment of the samples were avoided in both gels.

2.7 Zymogram assays

SDS-PAGE was performed as previously described (LaemmLi, 1970) and the supernatant samples were loaded to the gels. After electrophoresis, the gels were incubated for 4h at room temperature in several changes of renaturation buffer to obtain renaturation of enzymes. Renaturation buffer was 100 mM Tris/HCl, pH 7, containing 5 mM $MgSO_4$, and 1 % (v/v) Triton X-100. After the renaturation treatment, gels were equilibrated for 1 h in 100 mM sodium acetate buffer, pH 6 containing 5 mM $MgSO_4$, and then developed for phosphatase activity. For development, the gel was incubated at 37C^o for 1h in the same buffer used for equilibration with appropriate substrate added. Gels were washed in deionized water, and then soaked in different reaction buffers as a following procedure (Thaller et al, 1994). In the case of Native-PAGE, the electrophoresed gels were directly developed and soaked in the reaction buffers.

2.7.1 *p*-nitrophenylphosphate (pNPP) zymography

Acid Phosphatase activities in the gels were indicated by the presence of blue-stained bands. The gels are first developed with 5 mM (*p*NPP) in 25 mM sodium acetate buffer (pH 6.0) and then submerged in a freshly prepared reaction solution made by a 6:1 (v/v) mixture of acidified ammonium molybdate (4.2 g ammonium molybdate and 28.6 mL sulphuric acid) and 10% (w/v) ascorbic acid and incubated at 42 ºC for detection of the released inorganic phosphate (Thaller e al, 1994).

2.7.2 Fluorogenic substrate 4-methylumbelliferyl phosphate (MUP) zymography

In this assay I followed the method described by Kameshita et al. (2010) but with some modification. The original reaction mixture containing 50 mM Tris-HCl (pH 8.0), 0.1 mM EGTA, 0.01% (v/v)Tween 20, 2 mM dithiothreitol, 20 mM $MnCl_2$ and 0.5 mM MUP and the

modified reaction mixture containing 50 mM sodium acetate (pH 6), 0.01% (v/v)Tween 20, 20 mM MnCl2 and 0.5 mM MUP. Gels were incubated in 10 mL of the reaction mixture containing fluorogenic substrate as above for 15 min at 37°C, and fluorescent bands were observed by transilluminator FBTIV-88 (Fisher Scientific) with excitation wavelength at 365 nm. Longer incubation than 30 min is not recommended, since it will cause diffusion of fluorescent products formed in gels.

2.7.3 β – naphthyl phosphate zymography

For acid phosphatase activity the gels were incubated at room temperature in dark using 0.1 M sodium acetate buffer pH 6 containing 0.1 % β–naphthyl phosphate substrate. β–naphthol formed by the enzymatic hydrolysis of β–naphthyl phosphate was detected by post-coupling with 0.1% Fast blue BB salt or Fast Garnet GBC (SØRENSEN, 1972; Panigrahi, et al, 2007) (see Figure 4)

2.7.4 3,3,5,5-tetramethylbenzidine (TMBZ) substrate for Peroxidase activity

To determine the peroxidase activity, the enzyme assay was performed using 3,3,5,5-tetramethylbenzidine (TMBZ). Gels were immersed in a mixture of 4.2 mM TMBZ in methanol; 0.25 M sodium acetate (pH 5.0) (3:7, v/v). The mixture was incubated for 1h in dark then 0.5 mL of 30% H_2O_2 was added (SØRENSEN, 1972).

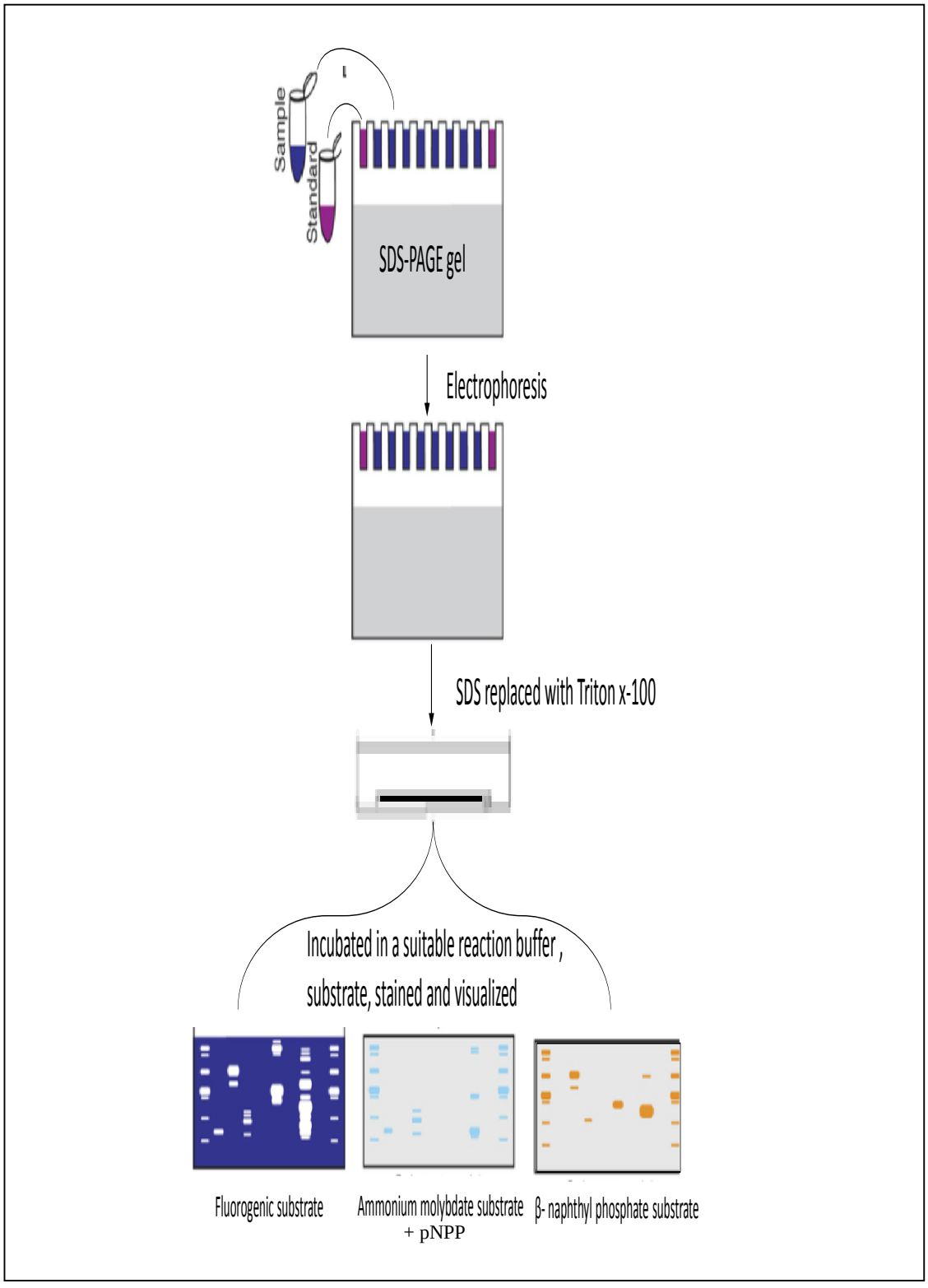

Figure 4. Zymography technique to assess the enzymatic activity of proteins by separating them with electrophoresis then the enzyme converts the substrate into a product which is detected by different staining methods.

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2.8 Purification of *S. albus* Acid Phosphatase (SapS) using Fast Protein Liquid Chromatography (FPLC)

Concentrated supernatant preparation (15 mL) was applied to a strong anion exchange (HiTrap Capto Q) column (GE Healthcare) equilibrated with 25 mM Tris-HCl buffer, pH 8.0. Unbound proteins were removed by washing the column with the equilibration buffer. Bound proteins were then eluted over stepwise gradient of 1M NaCl in 25 mM Tris-HCl buffer, pH 8.0. The active fractions of the eluate were pooled and concentrated through P-3 membranes in an Amicon ultrafiltration apparatus. The enzyme preparation was adjusted to pH 4 and applied to a cation-exchange (HiTrap Capto-S) column (GE Healthcare) equilibrated with 25 mM sodium acetate buffer, pH 4. The phosphatase was eluted with a 0-1 M linear NaCl gradient. The active fractions were pooled, and concentrated again by filtration through Amicon P-3 membranes and ready for SDS- PAGE analysis. Electrophoresis was carried out as described above on 12 %(w/v) acrylamide gels (SDS-PAGE) and then stained with either coomassie brilliant blue or silver stain.

2.9 In - gel protein digestions with subsequent identification by mass spectrometry

Trypsin was used for in-gel digestion. The following procedure for preparing gel slices for tryptic digestion started after de-staining gels and washing with 50mM ammonium bicarbonate (AmBic) for 5 min Then carefully cut the bands of interest using a scalpel and placed gel pieces in a siliconized eppendorf tube to reduces binding of the peptides to the tube surface which could interfere or suppress the MALDI-MS signal. Gel pieces were covered with 200 µl of 200 mM ammonium bicarbonate with 40% acetonitrile) and incubated at 37 ºC for 30 min. Solution

was removed and discarded from the tube and repeated one more time then dried the pieces in a Speed Vac concentrator (Fisher Electron Corporation, Savant 120) for15–30 min. 20 µl of the trypsin solution prepared (100 µl of 1 mM HCl to one 20 mg vial of trypsin (Sigma-Aldrich) + 900 µl of a 40 mM ammonium bicarbonate in 9% acetonitrile solution to the gel samples and 50 µl of 40 mM ammonium bicarbonate in 9% acetonitrile solution was added. Liquid from the gel pieces were removed and transferred to a new tube, after overnight incubation at 37 ºC. To increase the peptide yield by about 5%, 50 µl of a (0.1% trifluoroacetic acid (TFA) in 50% acetonitrile solution) was added to the gel pieces and incubated for 30 min at 37 ºC. The TFA solution then removed and combined with a solution containing the extracted tryptic peptides. The combined sample solutions were concentrated using Speed Vac concentrator and then sent to the Matrix-Assisted Laser Desorption Time-of-Flight (MALDI-TOF) mass spectrometry analysis in Western University and The Hospital for Sick Children, Toronto.

2.10 Amino-terminal amino acid sequencing

The purified proteins preparation was subjected to SDS-PAGE and transferred electrophoretically to a polyvinylidene difluoride PVDF membrane (Immobilon, Millipore). The transferred protein was stained with Ponceau S, and the band was excised and subjected to sequence analysis using gas-phase sequencer (PROCISE) at the Hospital for Sick Children analytical facility (Toronto, Canada).

2.11 Statistical analysis

For the statistical analysis and generation of graphs in this study, ANOVA with Prism 5 software (version 5.01; GraphPad Software Inc., CA) was used. The data were presented as mean ± standard deviation of three independent experiments performed in triplicate.

3. Results

3.1 Analysis of SapS activities in *S.albus* under conditions of different Pi availability

Bacterial acid and alkaline phosphatases are typically regulated by environmental phosphate concentration. The expression of these enzymes, which catalyze the hydrolysis of exogenous sources of phosphorylated components into inorganic phosphate, is induced under phosphate starvation. This essential limiting nutrient is then transported into the cell by membrane permeases. To determine if SapS is involved in phosphate assimilation, the effect of environmental phosphate concentration on SapS expression and activity were examined. *S.albus* were grown in Sauton's medium containing three different concentrations of inorganic phosphate, 0 , 0.2 and 2g/L, and the acid phosphatase activity of the culture filtrate protein (CFP) fractions and the OD were assayed in a time period of 10 days . The result showed that growth rate increased with increasing phosphate levels; a linear correlation was found with specific growth rate and Pi concentrations (Figure 5). However, SapS activity was severely decreased when inorganic phosphate (2g) was increased. High levels of acid phosphatase activity detected in the late log phase and peaked on day 7(Figure 6 and 7), confirming that SapS in *S.albus* is synthesized exclusively under Pi-limiting conditions.

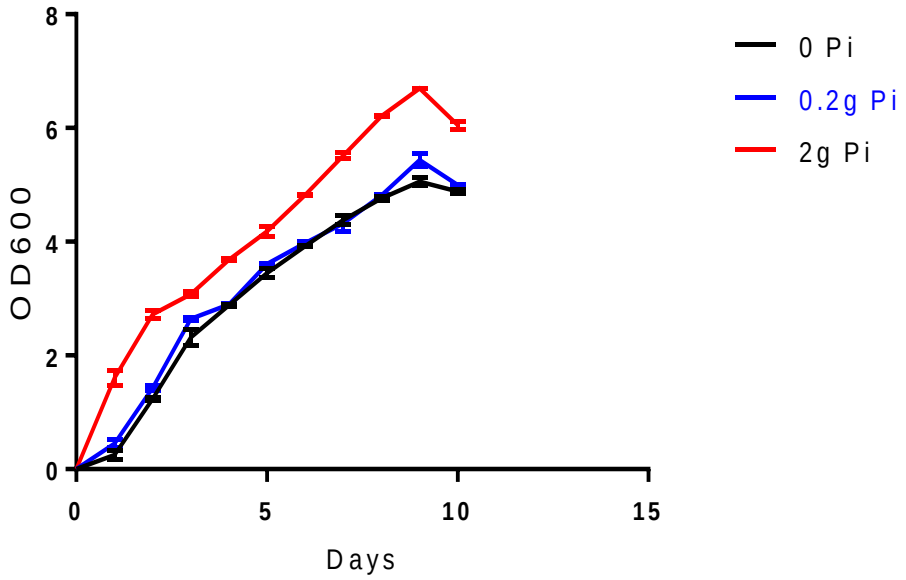

Figure 5. Growth kinetics of *S.albus* in Sauton's medium containing three different concentrations of inorganic phosphate, 0, 0.2 and 2g/L. The optical density of cultures grown were monitored for 10 days at absorbance (A600 nm). A linear correlation was found with specific growth rate and Pi concentrations. The values of absorbance are represented as the mean of three independent experiments carried out in triplicate.

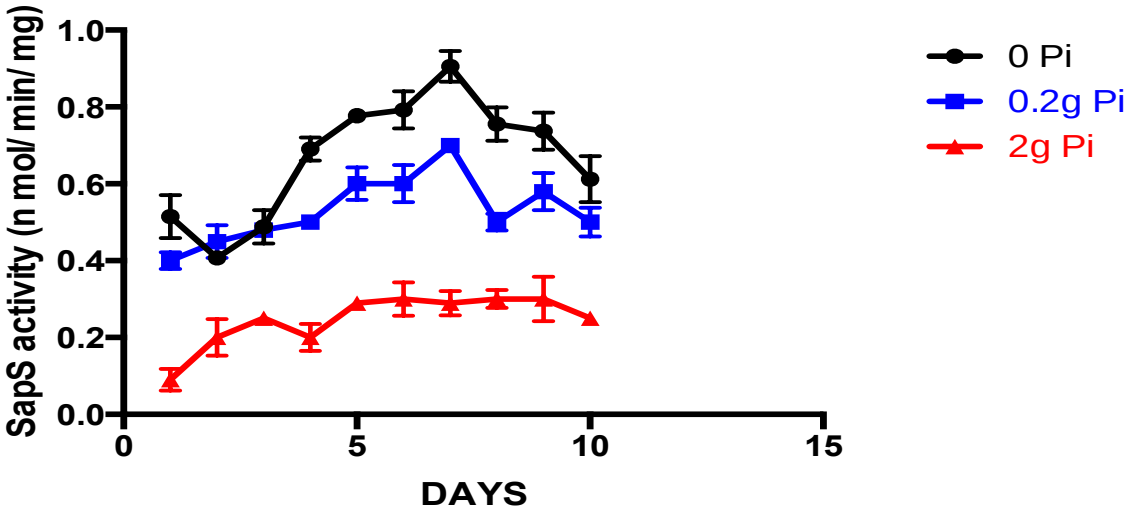

Figure 6. SapS activity of culture supernatant of *S. albus* was grown in Sauton's medium containing three different concentrations of inorganic phosphate, 0, 0.2 and 2g/L up to 10 days. Figure shows the effect of increasing concentrations of phosphate in the medium used to grow *S. albus*. Enzyme activities were determined every day as describe in methods and materials section. High levels of acid phosphatase activity detected in the late log phase and peaked on day 7 in phosphate starvation medium. The experiment was performed in triplicate and the values given are the averages.

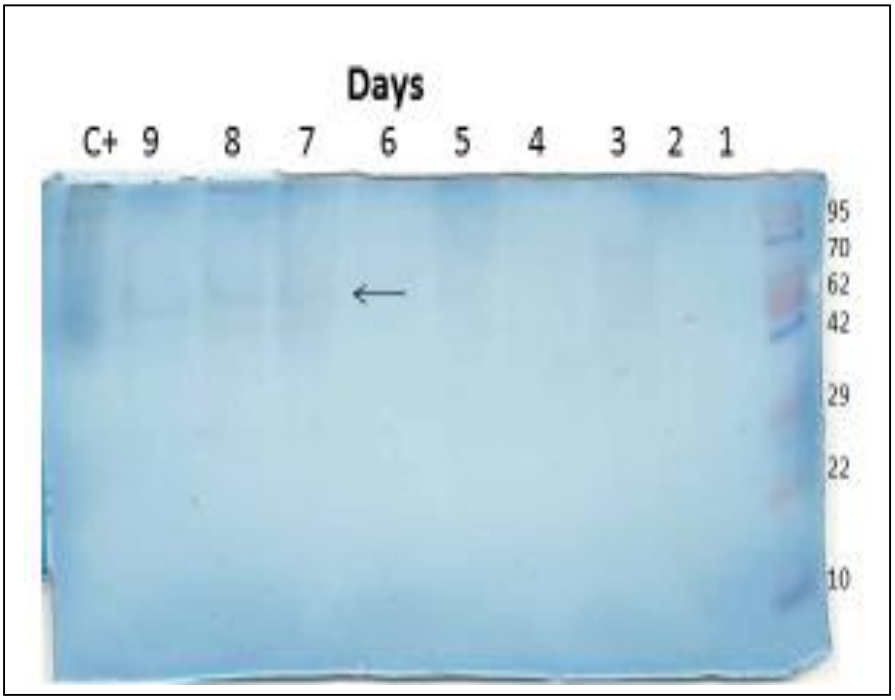

Figure 7. Zymogram developed for SapS activity after Native-PAGE using pNPP as a substrate. Lane1-9, 100µg of *S. albus* supernatant was loaded in each lane. Figure represents the high levels of acid phosphatase activity detected in the late log phase which became detectable by day 7 in phosphate starvation medium. (C+) is a wheat germ acid phosphatase as a positive control and protein size marker are reported in kDa on the right. These zymogram patterns were reproducible in triplicate experiments and representative results are shown.

3.2 Analysis of SapS activities in *S.albus* under conditions of different nitrogen source

Interestingly, almost the same levels of acid phosphatase activity were detected in *S.albus* grown in media containing different nitrogen source (ammonium chloride, glycine and sodium nitrate). The original Sauton's medium containing asparagine as the primary nitrogen source. Asparagine was substituted by these different sources at an equal molar concentration and the fresh medium was adjusted to pH7.4. The CFP fractions prepared under these conditions exhibited much higher levels of acid phosphatase activity than those grown in the original Sauton's medium (Table 1) and measuring the pH of these cultures media after growth revealed that growing cells leads to acidification of Sauton's medium containing NH_4Cl and $NaNO_3$. The pH changed from 7.4 to 6.3 and 4.6 respectively, whereas the pH of asparagine and glycine containing Sauton's medium remains at 7.2-7 (Table 1)

3.3 Determination of phosphatase activity of the supernatant and cell wall bound protein

To determine whether this activity was simply cell-wall associated phosphatase that was released by spontaneous lysis of some cells in the culture or other secreted bacterial phosphohydrolases, including alkaline phosphatases, supernatants and cell wall bound proteins were assayed for hydrolysis of pNPP at both pH 6 and 10. The supernatants showed a small amount of phosphatase activity at pH 10. However, the cell wall bound phosphatase , which were slightly rich in alkaline phosphatase, showed a minimal pNPP reaction at pH 6, indicating that alkaline phosphatase and cell wall associated phosphatase cannot be responsible for the activity in the supernatants of *S.albus* at pH6 (Figure 8).

Table 1. SapS activities were detected in *S.albus* grown in Sauton's medium containing different nitrogen sources (Ammonium chloride, Glycine, Sodium Nitrate and Asparagine) and pH adjusted at 7.4. Enzyme activity of SapS appears to increase as the pH of culture medium changes from 7.4 to 6.3 with the Ammonium Chloride as nitrogen source. Values given are the averages of at least three experiments.

Nitrogen source	**PH before the growth**	**PH after the growth**	**Acid phosphatase activity (nmol.min^{-1}.mg^{-1})**
L- Asparagine	7.4	7.1	1.9
Ammonium Chloride	7.4	6.3	2.9
Glycine	7.4	7	2.8
Sodium Nitrate	7.4	4.6	2.8

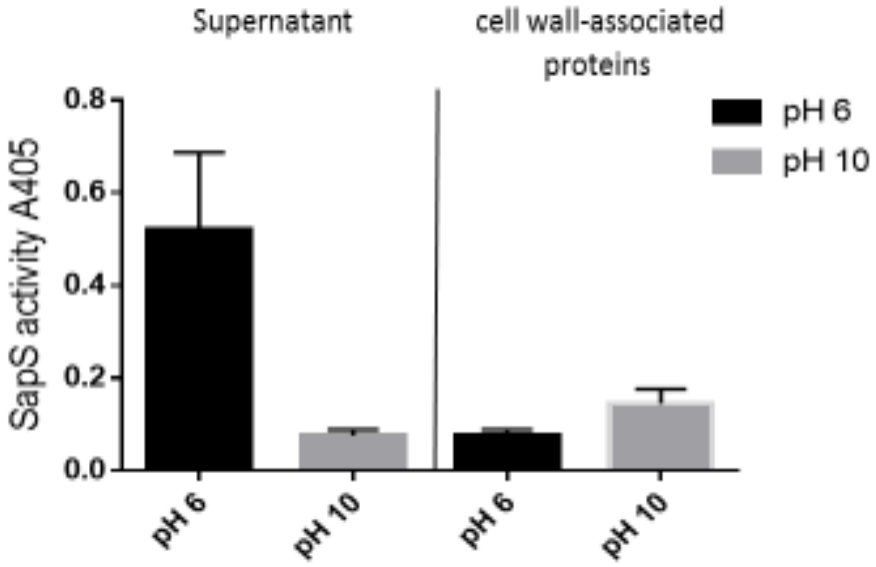

Figure 8. Acid and alkaline phosphatase activities in *S.albus* cultures. Supernatants and cell wall-bound proteins were examined for their ability to hydrolyze pNPP at pH 6 and 10. Bars represent the means ± standard deviation of three cultures. The only significant difference between them was observed with supernatants tested at pH 6.

3.4 Zymogram assays

A further alternative beyond enzymatic assays with crude preparations and enzyme purification is represented by the analysis of phosphatase activities using zymogram techniques. In this case crude extracts are first subjected to an electrophoretic separation, and phosphatase activities are subsequently detected in situ by means of chromogenic reactions. Such reactions are based either on substrates which yield colored products upon dephosphorylation or on the detection of the released Pi by means of the acidified ammonium molybdate method, which yields a blue precipitate or by means of the fluorogenic substrates that formed a fluorescent bands observed by transilluminator with excitation wavelength at 365 nm. Electrophoretic separation in zymograms is classically done under nondenaturing conditions, using gel electrophoresis. Although useful in separating the various activities, these procedures do not provide precise information on the molecular size of the enzyme. An interesting alternative is to conduct zymograms after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Rossolini et al 1998) followed by a treatment which allows enzyme renaturation in the gel matrix (renaturing SDS-PAGE) (Thaller et al, 1994). With this approach, proteins are separated on the basis of the size of their polypeptide component, and the migration distance at which the band of activity is detected depends on the molecular mass of the polypeptide component of the enzyme.

For the detection of SapS after electrophoresis, three different substrates were used. The choice of substrates for the detection of the acid phosphatase was based primarily on the results given by (Sorensen, 1972), and favored those substrates which showed the highest activity. The substrates were β–naphthyl phosphate, 4-methylumbelliferyl phosphate (MUP) and p-nitrophenylphosphate (pNPP).

3.4.1 In-gel acid phosphatase assay using β–naphthyl phosphate substrate

Firstly, the result of a zymogram performed following the electrophoretic run and developed at pH 6 using β–naphthyl phosphate as the substrate post – coupling with GBC and Fast Blue BB that incubated in dark at room temperature were two bands observed on the gels. Apparent molecular weight of the bands that observed for acid phosphatase with GBC post-coupling was estimated to be 18 kDa (Figure 9). However, detection of the substrate product by post-coupling with Fast blue BB salt showed a band estimated to be around 24-25 kDa (Figure 10).

3.4.2 In-gel acid phosphatase assay using pNPP substrate

Zymogram detection of acid phosphatase activities using pNPP substrate were performed essentially as previously described (Rossolini *et al.* 1994; Thaller *et al.* 1995) and SDS-PAGE was performed as previously described (LaemmLi 1970). Briefly, the protein preparations were subjected to SDS-PAGE and, after electrophoresis, the gel was incubated for 4 h at 37°C in several changes of renaturation buffer to obtain renaturation of enzymes. After the renaturation treatment, the gel was equilibrated for 1 h in several changes of equilibration buffer at the desired pH and then incubated at 37°C for 1 h in equilibration buffer containing the pNPP substrate. Finally the gel was thoroughly rinsed in distilled water and incubated at 42°C in a solution made by a 6:1 (v/v) mixture of acidified ammonium molybdate, for detection of the released inorganic phosphate. Using this procedure, phosphatase activities were indicated by the presence of two closely migrating blue-stained bands estimated to be around 72 and 50 kDa respectively (Figure11 C, lane1 and 2). In addition, crude supernatant that precipitated with ammonium sulfate showed a single band at a molecular weight of approximately 36 kDa (Figure 11C, lane 3).

In case of the native gel analysis under zymogram conditions, three bands were observed for acid phosphatase and their apparent molecular weight were estimated to 36, 50 and 72 kDa (Figure11B).

3.4.3 In-gel acid phosphatase assay using MUP substrate

In-gel acid phosphatase assay using fluorogenic substrate MUP was first examined after polyacrylamide gel electrophoresis in the absence of SDS, namely Native-PAGE. Varying amounts of supernatant were resolved on Native-PAGE at 4°C, and then the gel was incubated in the reaction buffers containing fluorogenic substrate. Activity showed two faint fluorescent bands around 36 and 50 kDa respectively (Figure12 A). Next, I examined denaturation/renaturation treatment before detecting acid phosphatase activity in SDS gel. The gel was incubated in the renaturation buffer at 4°C for 4h. After renaturation process, the gel was soaked in the reaction buffer containing MUP and gel showed just single clear fluorescent band from the concentrated sample with ammonium sulfate. It has estimated size of 36 kDa (Figure12 B).

The above data, along with results of zymogram activities, demonstrates that, Acid phosphatase activities on zymogram were semi quantitatively scored on the basis of the bands' intensities and the intensity gradually increased in parallel with the amount of sample loaded on the gels. These results indicated that *S.albus* may has at least two types of secreted acid phosphatase, one expected to be tetrameric protein (18, 36 and 72 kDa) and the other even expected to be dimeric protein (50 and 25kDa).

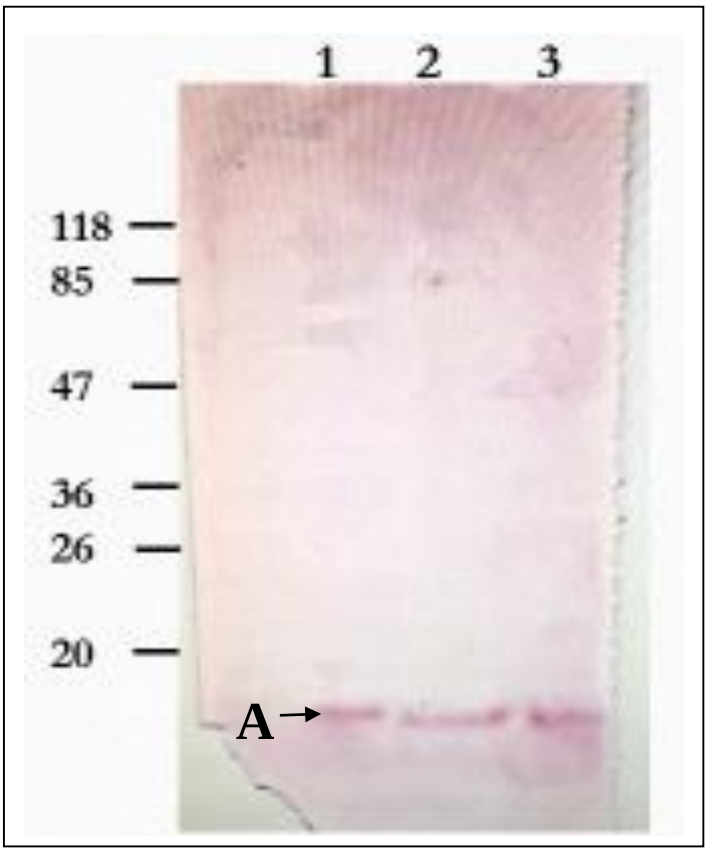

Figure 9. Zymogram developed for SapS activity after renaturing 14% SDS-PAGE using β–naphthyl phosphate as a substrate and post – coupling with GBC. *S. albus* supernatant were loaded with different amount of protein. Lane1, 40µg. Lane2, 100µg. Lane3, 100µg of the supernatant precipitated with ammonium sulfate. Protein size markers are reported in kDa on the left. These zymogram patterns were reproducible in triplicate experiments and representative results are shown. The band identified with arrow and labeled (A) refers to the protein identification that is shown in (Table 2).

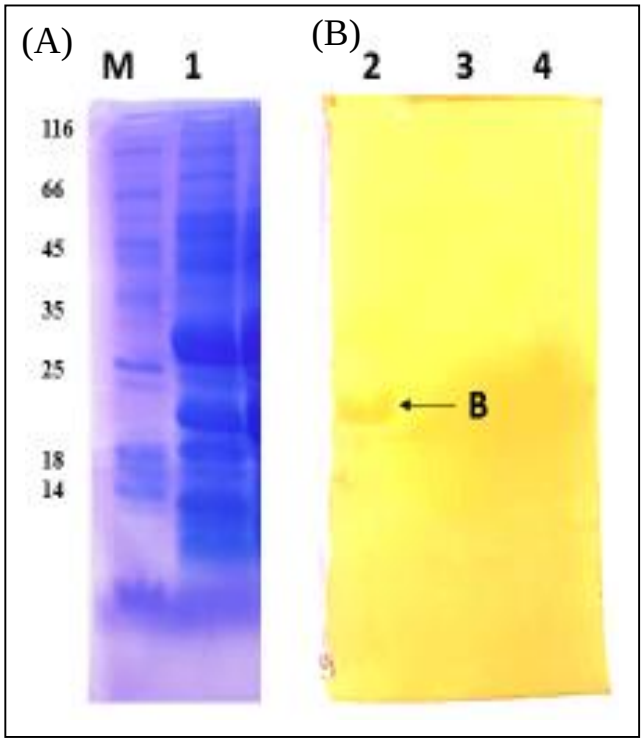

Figure 10. 12% SDS PAGE analysis of *S. albus* supernatant. (A) Lane 1, Coomassie blue stained crude supernatant. (B) Zymogram developed for SapS activity after renaturing using *β*–naphthyl phosphate as a substrate post-coupling with Fast blue BB. Lane2, 100µg. Lane3, 20µg. Lane4, 100µg of the supernatant precipitated with ammonium sulfate. Protein size markers are reported in kDa on the left. These zymogram patterns were reproducible in triplicate experiments and representative results are shown. The band identified with arrow and labeled (B) refer to the protein identification that is shown in (Table 3).

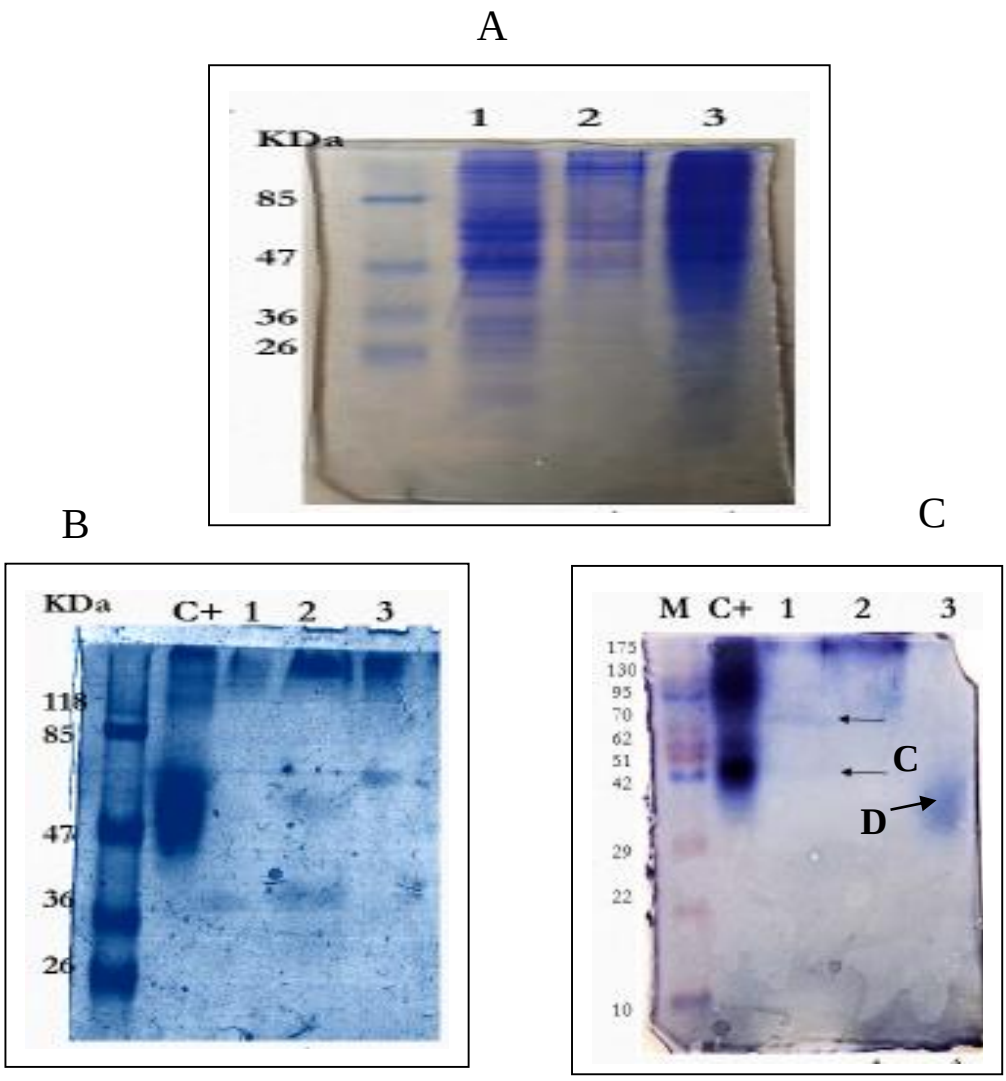

Figure 11. 12% polyacrylamide gel analysis of *S. albus* supernatant.(A) Coomassie blue stained crude supernatant after SDS-PAGE and different amount of protein were loaded (40 µg lane 1), (100 µg lane 2) and (100µg of supernatant precipitated with ammonium sulfate lane 3). (B&C) Results of zymograms performed for SapS activity following the electrophoretic run and developed at pH 6 using pNPP as substrate. Same samples as in (A) but in case of (B) a zymogram performed after native page and in case of (C) a zymogram performed after SDS-PAGE. (C+) is a wheat germ acid phosphatase as a positive control and protein size marker are reported in kDa on the left. The bands identified with arrows and labeled (C&D) refer to the proteins identification that is shown in (Table 4&5). These zymogram patterns were reproducible in triplicate experiments and representative results are shown.

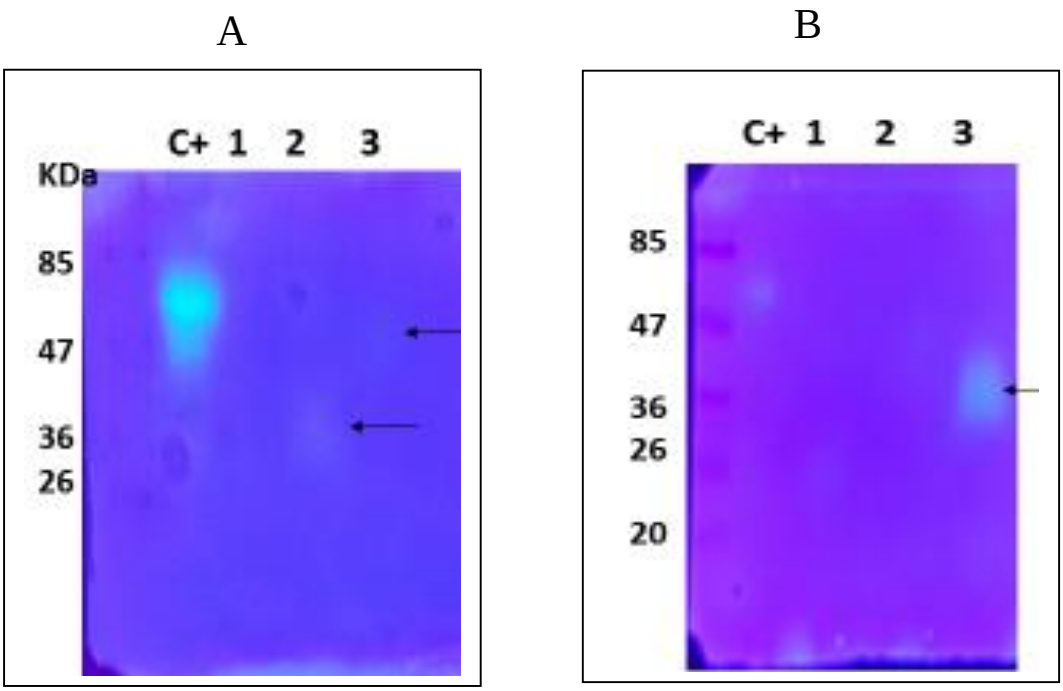

Figure 12. Zymograms performed for SapS activity following the electrophoretic run and developed at pH 6 using MUP as substrate**.** (A) 12% Native page analysis of *S. albus* supernatant. Lane1, 40µg. Lane2, 100µg. Lane3, 100µg of the supernatant precipitated with ammonium sulfate. (B) 12% SDS-PAGE analysis of *S. albus* supernatant and same samples as in (A). (C+) is a wheat germ acid phosphatase as a positive control and protein size marker are reported in kDa on the left. These zymogram patterns were reproducible in triplicate experiments and representative results are shown.

3.4.4 In-gel peroxidase assay using TMBZ substrate

To validate our zymogram technique, we choose another respiratory virulence factor that has been detected in TB with a similar pH range of activity as the acid phosphatase which is

peroxidase enzyme. To determine the peroxidase activity from the crude extract of *S.albus*, The enzyme assay was performed after non-denaturing polyacrylamide gel and SDS polyacrylamide gel using 3,3,5,5-tetramethylbenzidine (TMBZ) substrate. Gels were immersed in a mixture of TMBZ in methanol and sodium acetate (pH 5.0). The gels then were incubated for 1h in dark. In case of SDS page this procedure followed the renaturation process. A single band with activity was detected in native page with high molecular weight >45kDa (Figure13 A) whereas no result was detected in SDS gel. This band was then excised from the native gel and applied to SDS-PAGE gel. A single band of 14 kDa was found in the SDS-PAGE gel after Coomassie blue staining. (Figure13B).

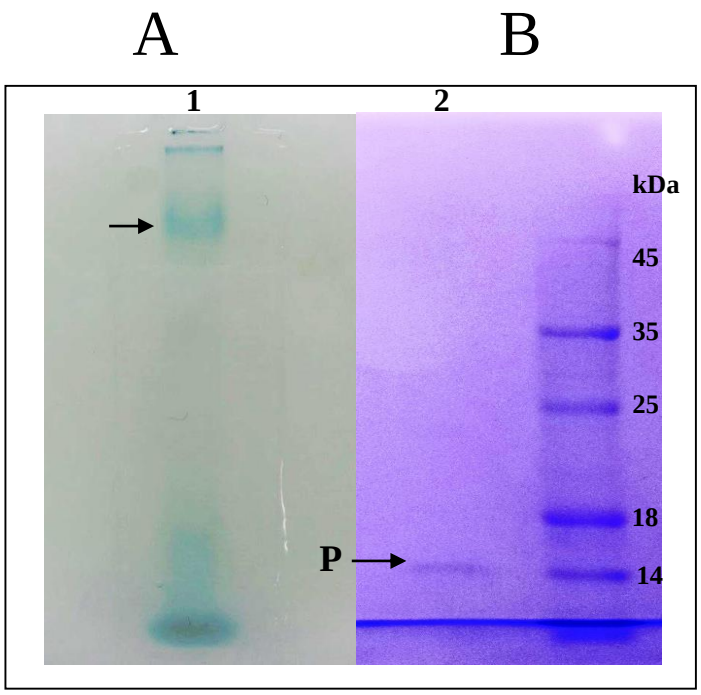

Figure 13. Zymograms performed for peroxidase activity using TMBZ substrate. (A) Result of a zymogram performed following 12 % Native page and developed at pH5 using (TMBZ) as substrate. Lane1, 20µg *S. albus* supernatant. (B) 12% SDS-PAGE analysis of the active band was excised from gel (A) and stained with Coomassie blue. Protein size markers are reported in kDa on the right. The band identified with arrow and labeled (P) refers to the protein identification that is shown in (Table 6). These zymogram patterns were reproducible in triplicate experiments and representative results are shown.

Table 2. MALDI-MS identification of Protein identified from zymogram using β–naphthyl + GBC

(Band **A** has expected size 18 kDa)

Top 5 candidate proteins identified from band A	Mass	Score	Matches	Protein function
*Inorganic pyrophosphatase	18853	18	40	Catalytic activity: Diphosphate + H(2)O = 2 phosphate.
Protein translocase subunit SecA	106505	27	60	Part of the Sec protein translocase complex. With the SecYEG preprotein conducting channel.
ATP-dependent Clp protease proteolytic subunit 2	24279	20	19	Cleaves peptides in various proteins in a process that requires ATP hydrolysis.
30S ribosomal protein S15	10784	20	12	One of the primary rRNA binding proteins.
4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase 1	40967	19	25	Catalytic activity.

* Indicates the most probable identification based on the molecular mass and the enzymatic activity observed.

Table 3. MALDI-MS identification of Protein identified from zymogram using β–naphthyl + Fast blue BB.

(Band **B** has expected size 24-25 kDa)

Top 5 candidate proteins identified from band B	Mass	Score	Matches	Protein function
* Ribonuclease H	25425	63	27	Catalytic activity: Endonucleolytic cleavage to 5'-phosphomonoester.
Chorismate synthase	41609	24	26	Catalytic activity: 5-O-(1-carboxyvinyl)-3-phosphoshikimate = chorismate + phosphate
Pyrophosphate--fructose 6-phosphate 1-phosphotransferase	36869	23	28	Catalytic activity: Diphosphate + D-fructose 6-phosphate = phosphate + D-fructose 1,6-bisphosphate.
Elongation factor Ts	29944	22	27	Associates with the EF-Tu. GDP complex and induces the exchange of GDP to GTP.
Tryptophan--tRNA ligase	37683	22	26	Catalytic activity: ATP + L-tryptophan + tRNA (Trp) = AMP + diphosphate + L-tryptophyl-tRNA(Trp).

* Indicates the most probable identification based on the molecular mass and the enzymatic activity observed.

Table 4. MALDI-MS identification of Protein identified from zymogram using pNPP and + ammonium molybdate detection system.

(Band **C** has expected size 50 kDa)

Top 5 candidate proteins identified from band C	Mass	Score	Matches	Protein function
* Ribonuclease H	25425	18	6	Catalytic activity: Endonucleolytic cleavage to 5'-phosphomonoester.
Inorganic pyrophosphatase	18853	20	5	Catalytic activity: Diphosphate + H(2)O = 2 phosphate
GTPase Era	34876	21	7	An essential GTPase that binds both GDP and GTP, with rapid nucleotide exchange. Plays a role in 16S rRNA processing and 30S ribosomal subunit biogenesis and possibly also in cell cycle regulation and energy metabolism.
UPF0109 protein SCO5592	8678	20	5	unknown
Lipoprotein CseA	23592	20	6	involved in the stabilization of the cell envelope

*Indicates the most probable identification based on the molecular mass and the enzymatic activity observed.

Table 5. MALDI-MS identification of Protein identified from zymogram using pNPP and + ammonium molybdate detection system.

(Band **D** has expected size 36 kDa)

Top 5 candidate proteins identified from band D	Mass	Score	Matches	Protein function
* Inorganic pyrophosphatase	18853	12	5	Catalytic activity: Diphosphate + H(2)O = 2 phosphate
Ribonuclease H	25425	22	7	Catalytic activity: Endonucleolytic cleavage to 5'- phosphomonoester.
30S ribosomal protein S18	9155	24	7	Binds as a heterodimer with protein S6 to the central domain of the 16S rRNA, where it helps stabilize the platform of the 30S subunit.
Probable RNA 2'-phosphotransferase	20403	21	6	Removes the 2'-phosphate from RNA via an intermediate in which the phosphate is ADP-ribosylated by NAD followed by a presumed transesterification to release the RNA and generate ADP-ribose 1"-2"-cyclic phosphate.
Oligoribonuclease	22122	20	5	3'-to-5' exoribonuclease specific for small oligoribonucleotides.

*Indicates the most probable identification based on the molecular mass and the enzymatic activity observed.

Table 6. MALDI-MS identification of Protein identified from zymogram using (TMBZ) substrate.

(Band **P** has expected size 14 kDa)

Top 5 candidate proteins identified from band P	Mass	Score	Matches	Protein function
*Superoxide dismutase [Ni]	14808	17	16	Catalytic activity: 2 superoxide + 2 H(+) = O(2) + H(2)O(2).
tRNA pseudouridine synthase A	31521	27	26	Formation of pseudouridine at positions 38, 39 and 40 in the anticodon stem and loop of transfer RNAs.
Ribosome-recycling factor	20774	20	24	Responsible for the release of ribosomes from messenger RNA at the termination of protein biosynthesis.
50S ribosomal protein L27	8769	19	14	Forms part of the ribosomal stalk which helps the ribosome interact with GTP-bound translation factors.
Peptide chain release factor 2	41117	19	20	Peptide chain release factor 2 directs the termination of translation in response to the peptide chain termination codons UGA and UAA.

*Indicates the most probable identification based on the molecular mass and the enzymatic activity observed.

3.5 Purification of SapS using FPLC

The results of the purification of secreted acid phosphatase activities from *S.albus* are summarized in (Table 7). Three enzymes were purified from the crude extract. The purification protocol involved two steps of chromatography. Four peaks of SapS activity were resolved on the strong anion exchange (HiTrap Capto Q) column, when washing the column with 25 mM Tris-HCl buffer (pH 8.0) and at 0.2 – 0.3 M NaCl concentration (Figure14). Active fractions were loaded onto a 12% SDS- PAGE and stained with silver stain (Figure15) and were assayed using zymogram after non-denaturing 10% PAGE by soaking the gel in buffer containing PNPP and developed with mixture of acidified ammonium molybdate as described previously . A single band was found in the highest active fractions number 1, SapS activity migrated slowly in the gel close to the 130 kDa marker (Figure16).

The peak of acid phosphatase resolved on the strong anion exchange column step was applied to a cation-exchange (HiTrap Capto S) column. Four peaks showing acid phosphatase activity (Figure17) were eluted and subjected to a zymogram (no band showed up) and SDS-PAGE staining with Coomassie brilliant blue (Figure18). Each pooled fraction showed a unique bands estimated to be around 25 kDa (Figure18 band F) and other two closely migrating bands were observed from active fractions 3 and 4 and their apparent molecular weight were estimated to 30-36 (Figure18 E) and 18 kDa (Figure18 G).

Table 7. Purification of *S. albus* secreted acid phosphatase using FPLC.

Purification Steps	Vol. (mL)	Protein (µg/mL)	Sp. Activity (nmol.min-1.mg-1)
Culture supernatant	500	435.657	0.576
Anion exchange column	15	888.912	0.184
Cation exchange column	3	88.282	1.120

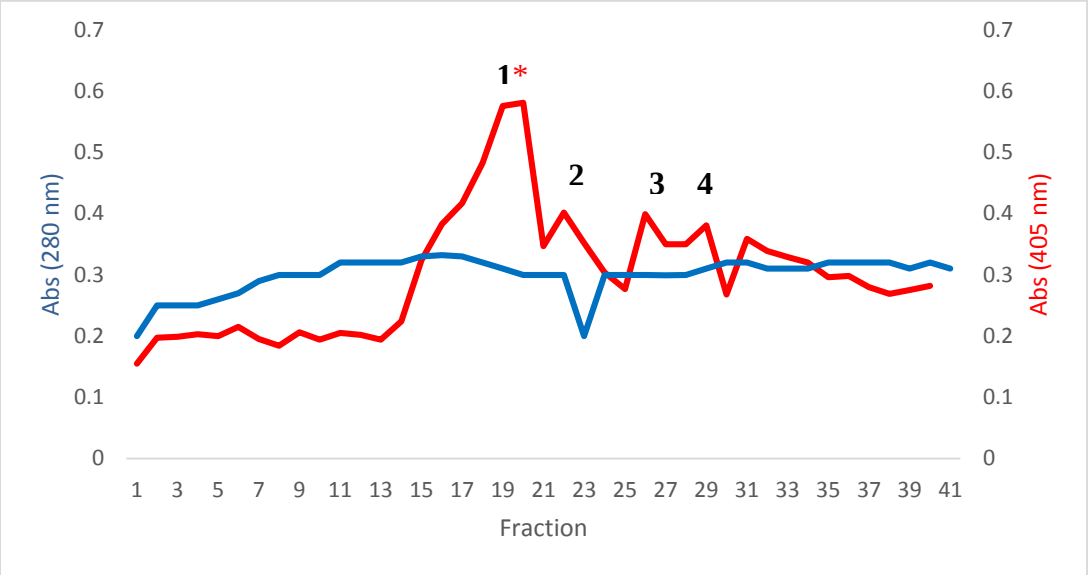

Figure 14. Elution of the SapS after purification of a culture supernatant by FPLC chromatography in a Strong anion exchange (Q) as described under "Experimental Procedures". Blue line is the Absorption at 280 nm; red line is SapS activity at 405 nm. Four pooled fractions referred by (1, 2, 3 and 4) found to contain SapS activity eluted between 0.2 – 0.3 M NaCl.

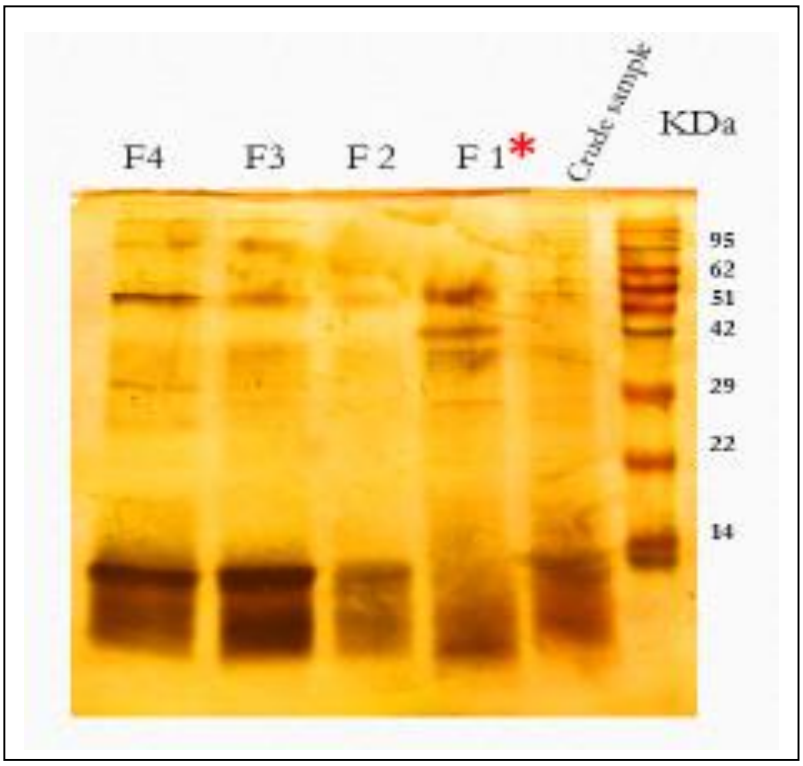

Figure 15. SDS-PAGE analysis of active fractions from the purification procedure using Strong anion exchange (Q) as described under "Experimental Procedures". The samples were loaded onto a 12% gel. Lane 1, crude extract of *S. albus*; F1, pooled fractions (15- 20) found to contain acid phosphatase activity; F2, pooled fractions (21-25); F3, pooled fractions (26- 30); F4, pooled fractions (31-35). Numbers on the right indicate the molecular mass (kDa) of the marker and gel stained with silver stain.

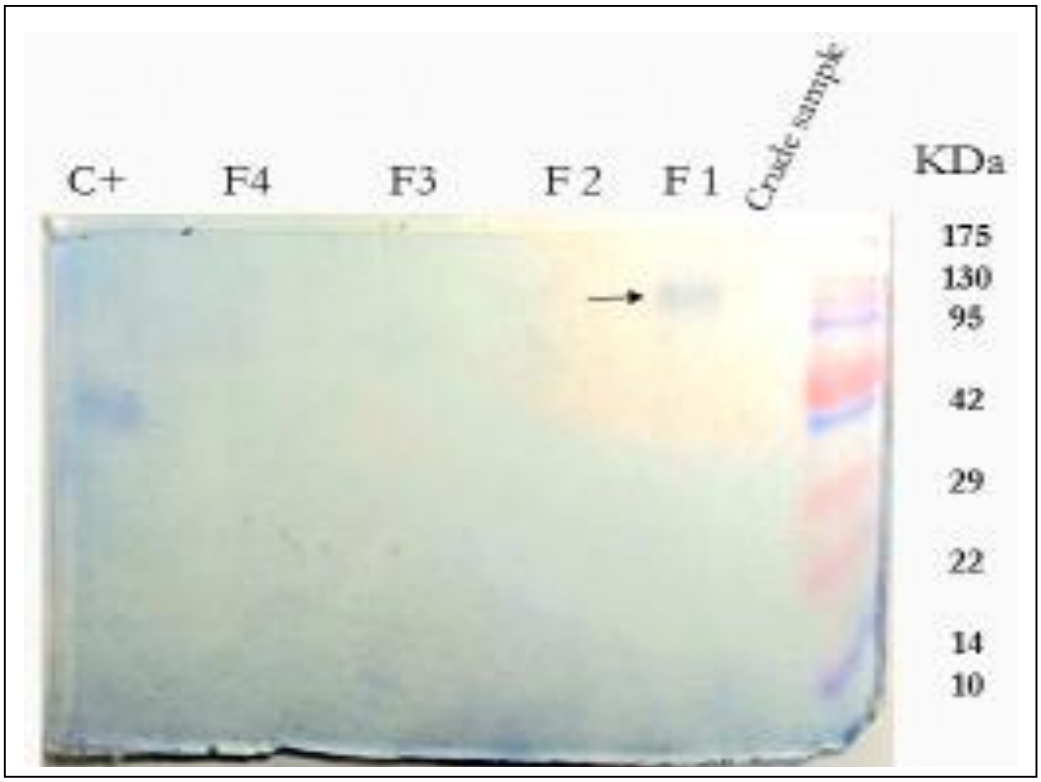

Figure 16. 10% Native–PAGE zymogram of active fractions after the purification procedure using Strong anion exchange (Q). After electrophoresis, the gel was developed for phosphatase activity against pNPP at pH 6. Lane 1, crude extract of *S. albus*; F1, pooled fractions (15- 20); F2, pooled fractions (21-25); F3, pooled fractions (26- 30); F4, pooled fractions (31-35). Numbers on the right indicate the molecular mass (kDa) of the marker. (C+) is a pure acid phosphatase as a positive control. A single band was found in F1which contain a highest acid phosphatase activity and no bands noticed in other fractions.

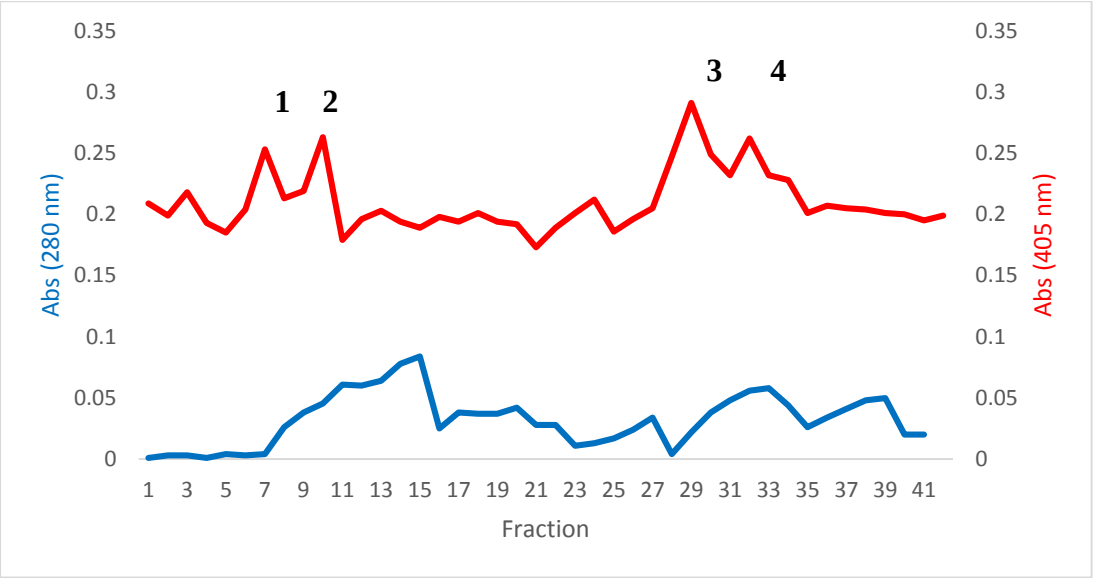

Figure 17. Cation-exchange (HiTrap Capto S) column FPLC chromatography of pooled and concentrated active fraction from anion exchange (Q). Application and elution of SapS was performed as described under "Experimental Procedures. Blue line is the Absorption at 280 nm; red line is SapS activity at 405 nm. Four pooled fraction referred by (1, 2, 3 and 4) found to contain SapS activity.

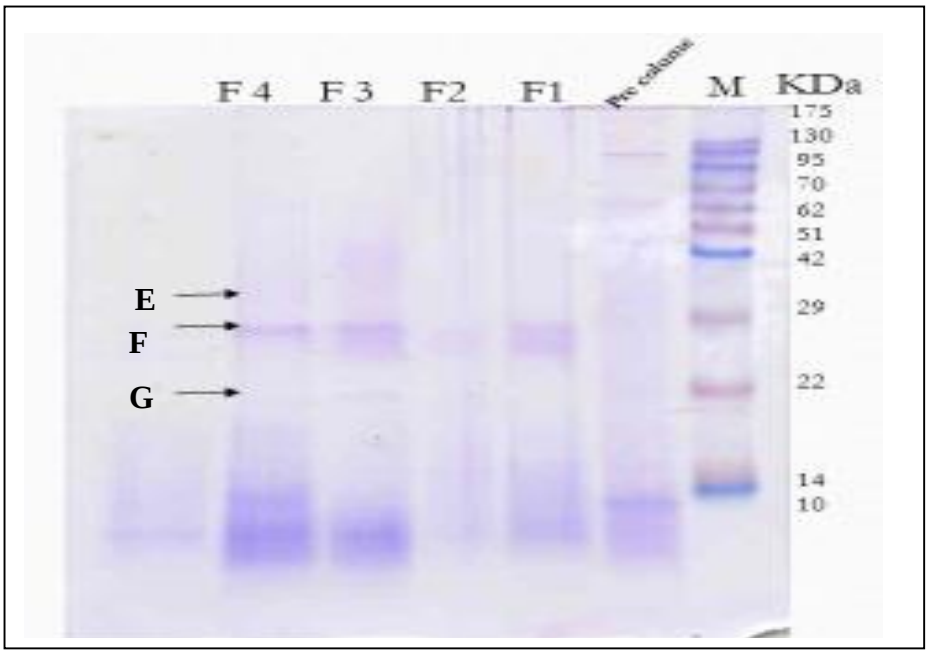

Figure 18. SDS-PAGE separation of active fractions from the purification procedure. From right to left: lane 1, molecular weight standards; lane 2, pre column of pooled and concentrated active fraction from anion exchange (Q). F1, pooled fractions (6-10) found to contain acid phosphatase activity; F2, pooled fractions (27-30); F3, pooled fractions (31- 35); F4, pooled fractions (36-40). Letters and arrows indicate the protein bands that were selected for tryptic digestion and identification using MALDI-TOF (Tables 8, 9 and 10).

Table 8. MALDI-MS identification of Protein purified from FPLC system.

(Band **E** has expected size 30-36 kDa)

Top 5 candidate proteins identified from band E	Mass	Score	Matches	Protein function
* Inorganic pyrophosphatase	18853	17	5	Catalytic activity: Diphosphate + H(2)O = 2 phosphate
Pantothenate kinase	37093	30	7	Catalytic activity: ATP + (R)-pantothenate = ADP + (R)-4'phosphopantothenate.
Histidinol dehydrogenase	46749	27	8	Catalyzes the sequential NAD-dependent oxidations of L-histidinol to L-histidinaldehyde and then to L-histidine.
Methionine import ATP-binding protein MetN	37272	27	8	Part of the ABC transporter complex MetNIQ involved in methionine import.
*Low molecular weight protein-tyrosine-phosphatase	17907	18	4	Catalytic activity: Protein tyrosine phosphate + H(2)O = protein tyrosine + phosphate.

*Indicates the most probable identification based on the molecular mass and the enzymatic activity observed.

Table 9. MALDI-MS identification of Protein purified from FPLC system.

(Band **F** has expected size 25-28 kDa)

Top 5 candidate proteins identified from band F	Mass	Score	Matches	Protein function
*Low molecular weight protein-tyrosine-phosphatase	17907	24	5	Catalytic activity: Protein tyrosine phosphate + H(2)O = protein tyrosine + phosphate.
4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	41169	24	7	Converts 2C-methyl-D-erythritol 2,4-cyclodiphosphate(ME-2,4cPP) into 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate.
HTH-type transcriptional repressor SCO4008	21259	23	6	Probably regulates the expression of its own gene and the adjacent SCO4007 gene by binding to two operator sites in the SCO4007-SCO4008 intergenic region.
tRNA-2-methylthio-N(6)-dimethylallyladenosine synthase	55747	22	9	Catalyzes the methylthiolation of N6-(dimethylallyl) adenosine (i(6)A), leading to the formation of 2 methylthio-N6(dimethylallyl)adenosine (ms(2)i(6)A) at position 37 in tRNAs that read codons beginning with uridine.
1-deoxy-D-xylulose-5-phosphate synthase 2	68744	20	10	Catalyzes the acyloin condensation reaction between atoms 2 and 3 of pyruvate and glyceraldehyde 3-phosphate to yield 1-deoxy-D-xylulose-5-phosphate (DXP).

*Indicates the most probable identification based on the molecular mass and the enzymatic activity observed.

Table 10. MALDI-MS identification of Protein purified from FPLC system

(Band **G** has expected size 18-21 kDa)

Top 5 candidate proteins identified from band G	Mass	Score	Matches	Protein function
*Low molecular weight protein-tyrosine-phosphatase	17907	34	5	Catalytic activity: Protein tyrosine phosphate + H(2)O = protein tyrosine + phosphate.
NADH-quinone oxidoreductase subunit I	23844	23	4	NDH-1 shuttles electrons from NADH, via FMN and iron-sulfur (Fe-S) centers, to quinones in the respiratory chain.
Probable allantoicase	41183	21	5	Catalytic activity: Allantoate + H(2)O = (S)-ureidoglycolate + urea.
UPF0109 protein SCO5592	8678	20	3	unknown
*Inorganic pyrophosphatase	18853	16	5	Catalytic activity: Diphosphate + H(2)O = 2 phosphate

*Indicates the most probable identification based on the molecular mass and the enzymatic activity observed.

4. Discussion

4.1 The secreted acid phosphatase are repressed by phosphate

To elucidate the biological function of SapS, we examined SapS expression under different stress. Bacterial acid phosphatases are regulated by environmental inorganic phosphate concentration (Thaller et al., 1994; Moura et al., 2001). In this study, we characterized the acid phosphatase activity produced by *S.albus* under conditions of different Pi availability, using pNPP as substrate, in culture filtrate preparations. In Pi-starved cultures with no addition of any organic phosphate source, high-level phosphatase activity at acidic pH values was obtained and a peak of phosphatase activity appeared in the late log phase at day 7. However, a much lower phosphatase activity, as compared to the above, when cells were grown in the medium containing abundant Pi (see Figure 6), which is in agreement with the result obtained in the previous study that showed growth of *Streptomyces griseus* on media containing Gylcerol-2-phosphate as the sole phosphate source caused a moderate increase of phosphatase activity, while Pi-starvation with no available organic phosphate source caused a remarkable increase of phosphatase activity (Moura et al., 2001).

In our study, we selectively chose Sauton's media for *S. albus* growth because it is well defined, minimal, and protein/peptide free so as to facilitate proteomic analysis in the extension of this study and it was developed originally for *M. tuberculosis* (Sassetti et al., 2003).

4.2 SapS is selectively expressed at mildly acidic pH

Although our initial hypothesis was that SapS is regulated by the availability of nitrogen source, acid phosphatase activity was assayed in four culture media having four different nitrogen sources (Sauton's medium with the nitrogen source being either ammonium chloride, asparagine, nitrate, or glycine) and the pH were measured before and after the growth as well.

The result indicated that the activities were almost similar except for asparagine which was the lowest. On the other hand, a very slight increase of the activity with the ammonium chloride source, the most likely reason for this observation is the acidification of the culture media. Indeed, measuring the pH of the culture media indicated that cell growth leads to acidification of Sauton's media containing NH_4Cl and nitrate (i.e., the pH changed from 7.4 to 6.3 and 4.6 respectively) ,whereas the pH of asparagine and glycine containing Sauton's media remain at 7 (Table 1). The most likely reason for these observations is the excretion and accumulation of organic acids during stationary and late phases of growth. Nitrogen metabolism in streptomyces has been well defined, nitrogen regulated genes (GlnR regulon) that controls the expression of three key enzyme systems involved in nitrogen assimilation. These three enzymes assimilate inorganic nitrogen (nitrate or ammonium ion) into organic form, so under high inorganic nitrogen conditions, as was used in this study, there will be accumulation of several organic acids (Voelker and Altaba, 2001; Madden et al., 1996). In addition, uptake of ammonia (NH_4^+) is accomplished by a membrane transporter, which may couple ammonia uptake to H^+ export (Westhoff et al., 2002). This would result in a gradual acidification of the culture medium. These results, taken together, suggest that secretion of the acid phosphatase activity correlate with acidification of the media not dependent on nitrogen sources, and a maximal level of acid phosphatase activities were detected at pH 6.3 under mildly acidic condition (i.e., pH 5.5-6.5) and that was confirmed in this study. This drop in pH was also observed when *M. tuberculosis* and *M. bovis* BCG are cultured in Sauton's medium containing ammonium chloride as a nitrogen source, and indicated that the acidification of the culture medium is responsible for the induction of SapM expression under mildly acidic at optimum pH 6.4 (Saleh and Belisle, 2000), that is consistent with mycobacteria containing phagosomes (Sturgill et al., 1994).

A last important observation of SapS characterization was that the higher enzymatic activities of SapS which were found in the supernatant fractions under acidic pH 6 comparing with cell wall-associated proteins. Otherwise, a very small amount of phosphatase activity was found in the supernatant and cell wall-associated proteins fractions under alkaline condition pH 10, indicating that SapS is indeed a secretory enzyme and the observed phosphatase activity is not simply found in this fraction as a result of leaking cell wall-associated phosphatase to the external medium or experimental error or cross contamination of the fractions during sample processing. These differences were confirmed by examining the SapS activity on the supernatant and cell wall-associated proteins fractions under alkaline and acidic conditions (Figure 8).

4.3 Zymogram analysis of SapS

To analyse in more detail the secreted acid phosphatase activities produced by *S.albus* under the above culture conditions, zymograms were performed on crude supernatant protein preparations using three different substrates *β*–naphthyl phosphate ,pNPP and MUP . This analysis showed that, with using *β*–naphthyl phosphate as a substrate, two active bands composed of 18 and 25 kDa (Figure 9&10) polypeptide units, respectively, were produced under the same conditions but with different salt coupling, while using pNPP activity, bands constituted by a 72, 50 & 36 kDa polypeptide units (Figure 11) and the same polypeptide units were produced 50 & 36 kDa (Figure 12) when using MUP substrate. Both Native and SDS-PAGE techniques were attempted in this study. Although electrophoretic separation in zymograms is classically done under non denaturing conditions and useful in separating the various activities, the renaturing SDS-PAGE technique was preferred to native electrophoresis techniques since it allowed a good resolution of activities, providing at the same time information on the molecular mass of the polypeptide which constitutes the protein. The fact

that we were able to detect different phosphatase active bands on gels and with no significant migration distance at which the bands of activity were noticed between the Native and the SDS gel, in zymograms should not be surprising since the different patterns of phosphatase activity observed in the present study may reflect the differences in the substrate specificities of different phosphatases (Panigrahi, 2007). Moreover, the limitations of SDS-PAGE to separate homopolymeric enzymes are a result of the avoidance of boiling and 2-mercaptoethanol addition in this study which would cause irreversible denaturation of the protein (which is very important step with the presence of SDS for protein separation) (Rossolini et al., 1998). In addition, some classes of phosphatase enzymes tend to be quite resistant to depolymerization by SDS and, in SDS-PAGE, migrate at least in part as their native form (homotetrameric proteins),if the sample, prepared in LaemmLi's buffer (LaemmLi, 1970), is not subjected to the boiling treatment (Thaller et al.,1995; Uerkvitz, 1988; Thaller, 1997). It should finally be noted that the use of a renaturing SDS-PAGE technique could have also resulted in missing enzymatic activities which are not able to renature following SDS-PAGE (Thaller et al., 1994*).*

The choice of substrates in this study for the detection of the acid phosphatase was based primarily on the results given by (Luffman and Harris, 1967), and favored those substrates which showed the highest activity. The substrates were phenolphthalein diphosphate, p-nitrophenyl phosphate (pNPP), 5-Bromo-4-chloro-3-indolyl phosphate (BCIP), lead sulfide, *β*-naphthyl phosphate and 4-methylumbelliferyl phosphate (MUP). Only three of these substrates gave an informative zymogram: pNPP, MUP and *β*-naphthyl phosphate while with phenolphthalein diphosphate, lead sulfide and BCIP non-informative bands were produced. Almost a similar zymogram pattern was obtained when using pNPP and MUP as substrates, as judged by inspection, higher intensity of the pNPP zymogram as compared to the same components in the MUP pattern. Furthermore, the formulae of the two substrates (pNPP & *β*-naphthyl phosphate) are aromatic compounds, but *β*-naphthyl phosphate has no substituents in

addition to the ester group, while p-nitrophenyl phosphate has an additional group, so, the substituents are attached to the carbon atom in the para position relative to the ester group. It was suggested that this may cause the different patterns of the pNPP and *β*-naphthyl phosphate zymograms (Sørenson, 1972). It was reported that if a substituent in *β*-naphthyl phosphate is attached to the carbon atom adjacent to the ester group, no enzymatic activity can be detected. Furthermore, the addition of a nitro group to phenyl phosphate has great influence on the activity of the enzyme, depending on which carbon atom relative to the ester group was the site of the substitution (Sørenson, 1972).

In addition to the enzymatic activities the acid phosphatases were analyzed for several different properties including the MW of the polypeptide, substrate specificity and preferred pH for activity by means of zymographic techniques and MALDI-MS identification. The deduced polypeptide of the *S. albus* proteins identified from mass spectrometry data were compared to all proteins sequences of *Streptomyces coelicolor* present in the SwissProt database by means of the EXPASY (Mascot) program. According to the proteins identified in zymograms assay taking into consideration the mass size, score and the function of each candidate proteins (Table 2,3,4&5), could be classified into two results: 1) bands A & D (see Figure 9,11&12) both giving a significant similarity to Inorganic pyrophosphatase protein (Table 2&5) , and result 2), bands B & C (Figure10 &11) both giving a significant similarity to Ribonuclease H (Table 3&4). Bands A and D showed 60% and 22% sequence identity to that of *S. coelicolor* Inorganic pyrophosphatase respectively, this enzyme catalyzes the conversion of one molecule of pyrophosphate to two phosphate ions . Previous studies confirmed the ability of Inorganic pyrophosphatase to hydrolyze a chromogenic substrates for most phosphatases include *β*–naphthyl phosphate, pNPP (Heinonen and Lahti, 1981; Curdova et al., 1982) and even the florigenic substrate like MUP (Nannipieri et al., 2011). The observation of the differences of enzyme sizes in this result may indicated that the enzyme

monomer has a molecular size of ~18 kDa and is believed to function as tetramer (36 and 72kDa) as reported in many previous studies, NSAPs are usually homotetrameric proteins comprising four polypeptide subunits in their native form (Rossolini, 1998). For result 2, band B and C shown 74% and 15% matched peptides to *S. coelicolor* Ribonuclease H respectively. Since these band indicated as RNase H and also exhibited acid phosphatase activity with different substrates , I proposed this enzyme is to similar to the SCO2299 gene from *S. coelicolor* which encodes a single peptide consisting of 497 amino acid residues and also encodes a bifunctional enzyme consisting of the RNase H domain and the acid phosphatase (APase) domain. Moreover, there results indicated that RNase H and acid phosphatase activities of the full length SCO2299 protein depend on its N-terminal and C-terminal domains, respectively. The relation between RNase H and acid phosphatase remain to be determined. However, the bifunctional RNase H/Acid phosphatase enzyme is a novel member in the RNase H family. Additionally, *S. coelicolor* is the first example of an organism whose genome contains three active RNase H genes (Ohtani et al., 2005). In addition, this enzyme monomer may have a molecular size of ~24-25 kDa and is believed to function as a dimer (24-25and 48-50kDa).

Microorganisms such as E.coli and yeast have well characterized systems involving many enzymes acting together to release phosphate from organic sources and to transport the solubilized phosphate efficiently into the cell. Along with the acid phosphatase, suspension cultures of these cells produce an extracellular RNase in response to phosphate starvation (Nurnberger et al., 1990). When phosphate-starved cells are returned to medium containing phosphate, no further synthesis of RNase occurs, indicating that the expression of RNase in this case plays a role as the acid phosphatase (Glund et al., 1993). RNase is thought to function as a phosphate –scavenging enzyme (D'Alessio & Riordan, 1997).

Finally, The results of multiple sequences alignment analysis (MULTALIN multiple alignment- EXPASY) (Corpet, 1988) between the identified proteins and *Mycobacterium tuberculosis* , showed a significant degree of sequences homology between these two family, i.e. the overall amino acid identity was 68% when the inorganic pyrophosphatase was aligned with *M. tuberculosis* inorganic pyrophosphatase (gi|625006479) (Figure 19) and the alignment between RNase H sequence and *M. tuberculosis* was similar to acid phosphatase (gi|685996982) with 33% identity (Figure 20) and also similar to bifunctional RNase H/acid phosphatase (gi|339298765) 33% identity (Figure 21).

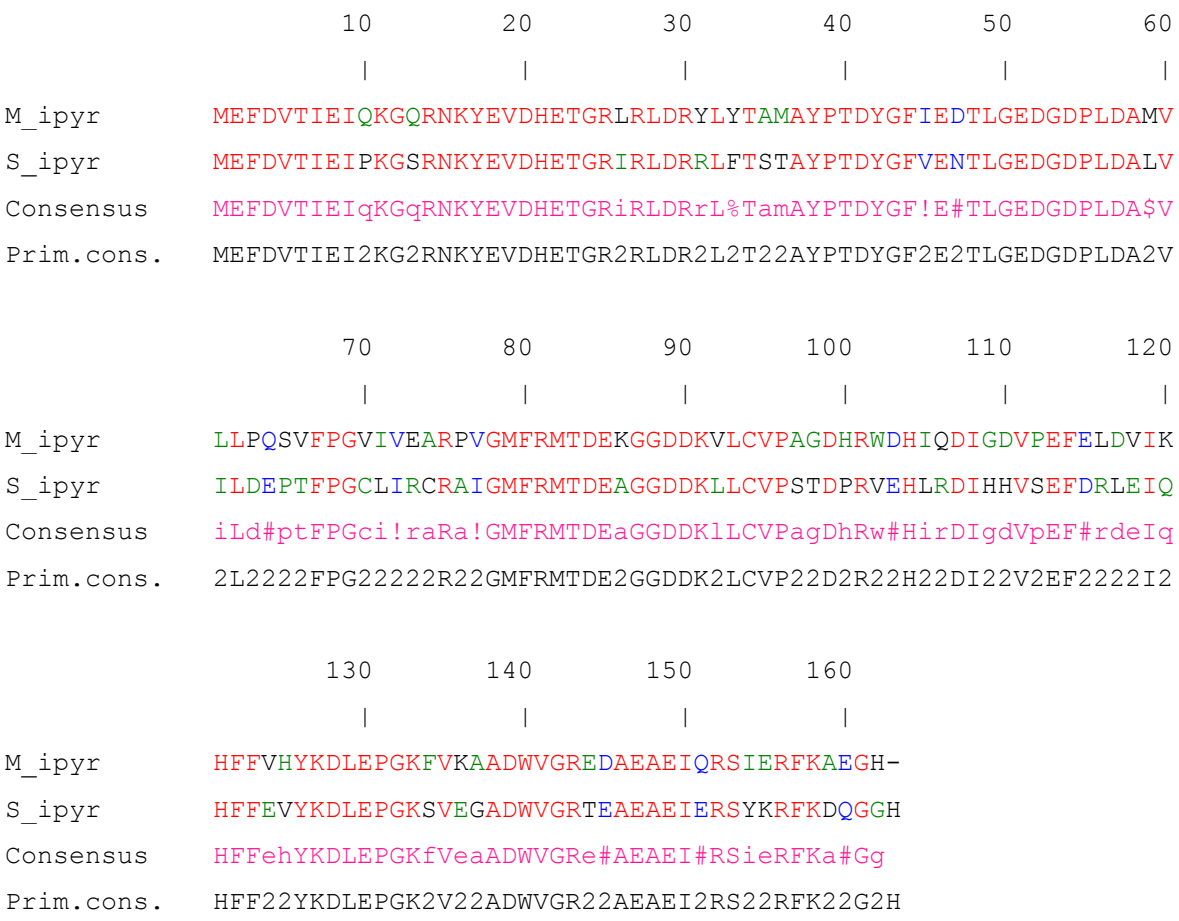

Figure 19. Amino acid sequences alignment of *S. coelicolor* inorganic pyrophosphatas (S_ipyr) with *M.tuberculosis* inorganic pyrophosphatase (M_ipyr). Alignment data: Alignment length 162. Identities 68%. Positives 82%.

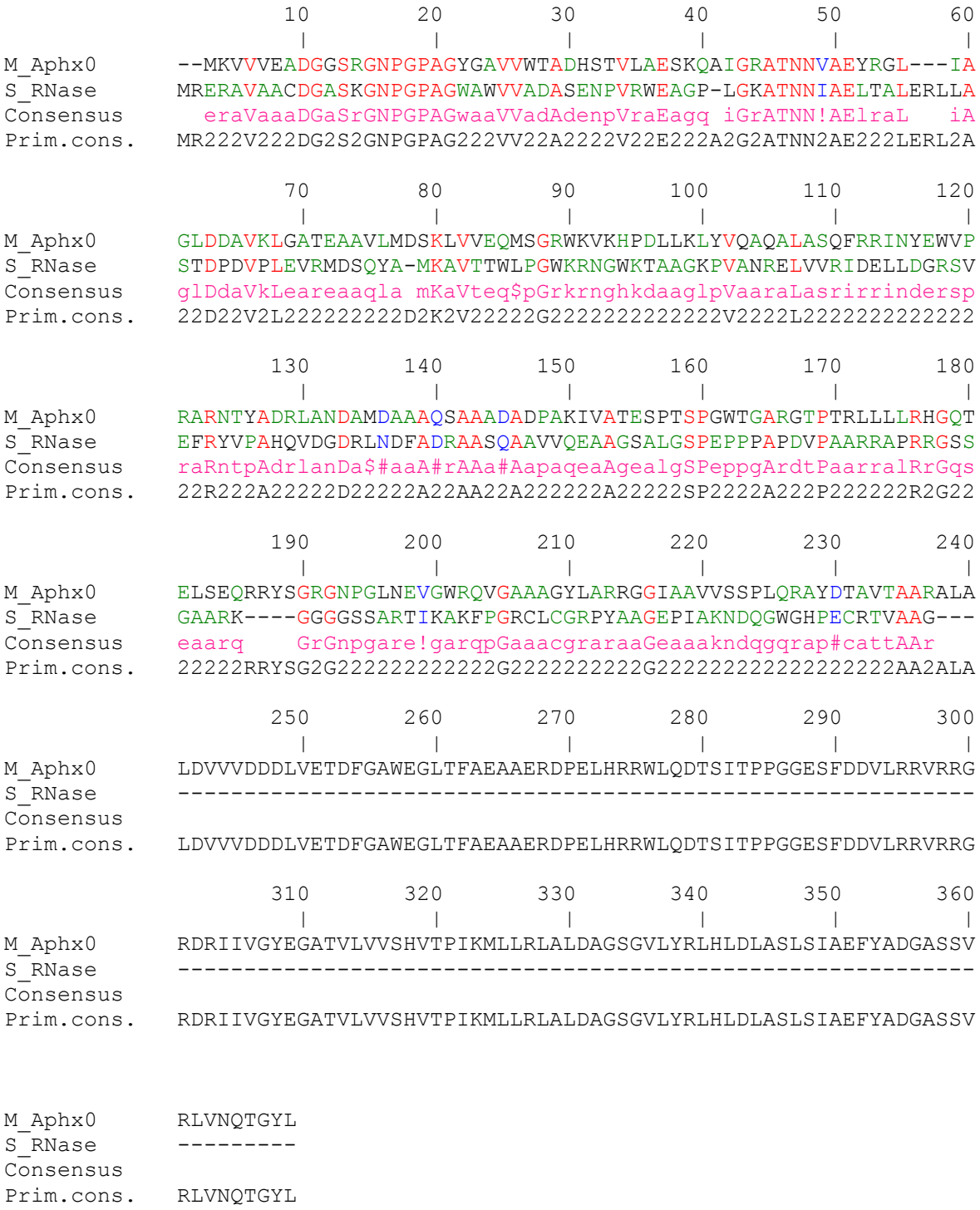

Figure 20. Amino acid sequences alignment of *S. coelicolor* RNase H (S_RNase) and *M.tuberculosis* acid phosphatase (M_Aph). Alignment data: Alignment length 364. Identities 33%. Positives 46%.

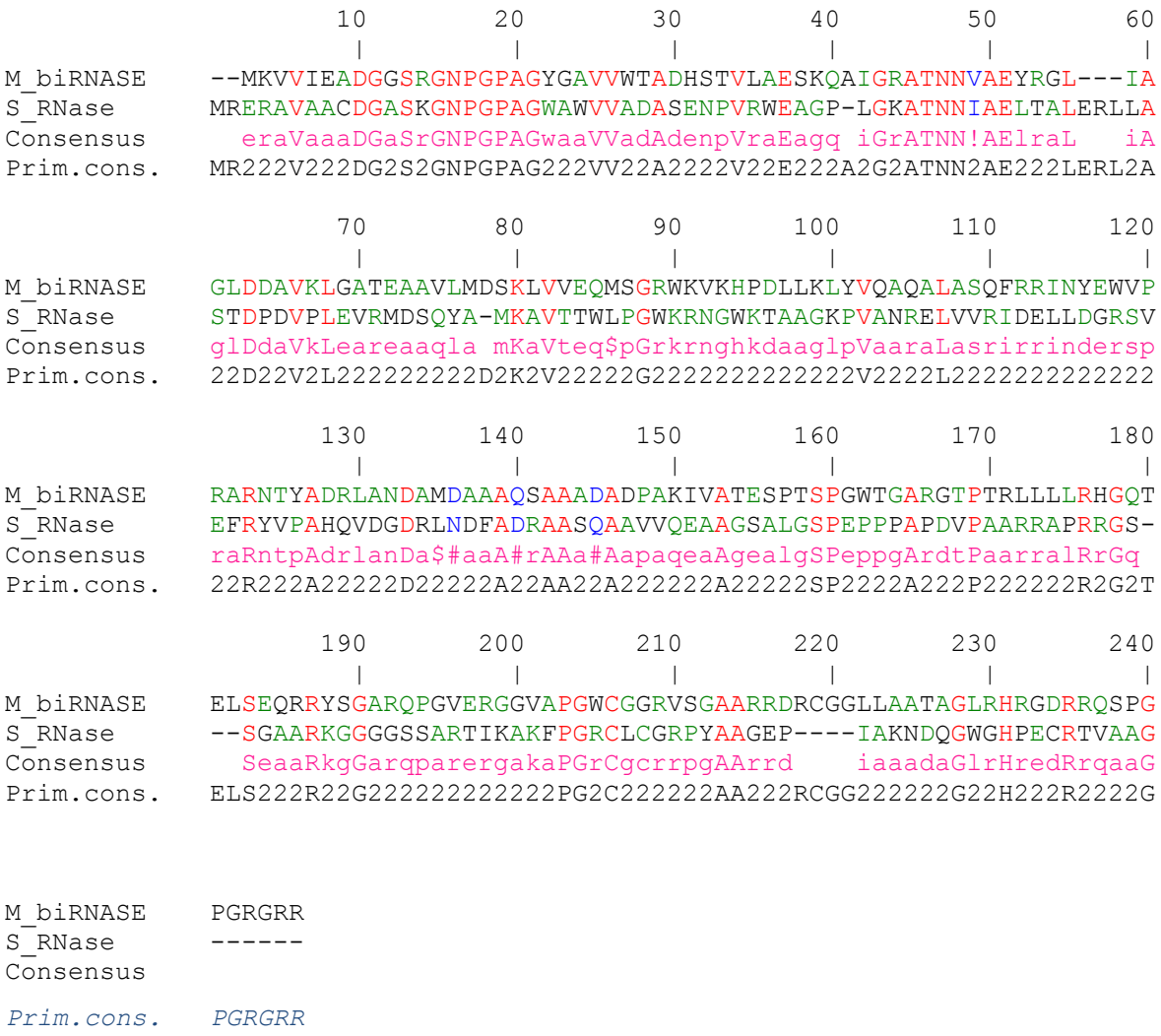

Figure 21. Amino acid sequences alignment of *S. coelicolor* RNase H (S_RNase) and *M.tuberculosis* bifunctional RNase H/acid phosphatase (M_biRNASE). Alignment data: Alignment length 241. Identities 33%. Positives 46%.

4.4 Purification of SapS enzyme using FPLC

Purification of SapS produced by *S.albus* was obtained in two steps using ion exchange columns chromatography (Figure 18, see Methods for details on the purification procedure). The specific activity of the purified protein, assayed against pNPP at pH 6, was 1.120 nM.min^{-1}.mg^{-1}. The N-terminal sequence of the purified proteins (band F and G) were N-terminally blocked (no sequences were obtained) (see appendix 1). The molecular mass of the three proteins bands (E,F&G) estimated, were approximately 30-36, 25,and 18-21 kDa respectively (Figure 18). This finding was confirmed by MALDI identification, which showed that band E has 20% and 10 % similarity to that of *S. coelicolor* Inorganic pyrophosphatase and Low molecular weight protein tyrosine phosphatase respectively (see table 8). Band F showed 15% homology to *S. coelicolor* low molecular weight protein tyrosine phosphatase (Table 9). Furthermore, band G showed a similar homology to band E, 30% matched peptide to *S. coelicolor* Low molecular weight protein tyrosine phosphatase and 25% to *S. coelicolor* Inorganic pyrophosphatase (Table 10). In (Li & Strohl, 1996) study, they described the isolation and characterization of a gene protein tyrosine phosphatase (*ptpA*) from *Streptomyces coelicolor* A3 that codes for a protein with a deduced *M*r of 17,90 containing significant amino acid sequence identity with mammalian and prokaryotic small, acidic phosphotyrosine protein phosphatases (PTPases) and they found that the purified fusion enzyme catalyzed the removal of phosphate from *p*-nitrophenyl phosphate (pNPP), The pH optima for pNPP hydrolysis by PtpA was 6.0 and the biological functions of PtpA and its putative homologs in Streptomycetes are not yet known. Moreover, in there experiment they observed that, the molecular weight of the purified polypeptide as measured by SDS-PAGE was bigger than that predicted from the sequence of the fusion protein (predicted *M*r, 18,258) and what observed was polypeptide with an *M*r of about 21,000 which is very close to our result in this study (see Figure 18 band G). However, in the (Koul et al, 2000) study, they reported the cloning and characterization of two

genes with sequence homology to those encoding protein tyrosine phosphatases that were cloned from genomic DNA of *Mycobacterium tuberculosis* H37Rv. The calculated molecular masses of these two putative tyrosine phosphatases were 17.5 and 30 kDa, respectively. In addition, they showed that these phosphatases are secreted into the culture medium and the comparison of these enzymes with other known tyrosine phosphatases showed the alignment with LMW phosphatases from *Streptomyces coelicolor* (PTPA) and also our MULTALIN search in this study reveals a significant degree of alignment to *M. tuberculosis* , 68% identity when the inorganic pyrophosphatase was aligned with *M. tuberculosis* inorganic pyrophosphatase (gi|625006479) (Figure 19) and 50% alignment between Low molecular weight protein tyrosine phosphatase sequence and *M. tuberculosis* protein tyrosine phosphatase (gi|686037535) (Figure 22). Finally, regardless of the protein being a pyrophosphatase or tyrosine phosphatase, they are both considered as virulence factors based on the knowledge of phosphatase function in other pathogens. It has been suggested that these enzymes may play an important role in the pathogenicity of mycobacteria by interfering with phosphotyrosine-mediated signals in macrophages. Even more, this result confirms the findings reported previously from our laboratory (Aljassim, 2015) in which immunoblotting with seven monoclonal antibodies from *M. tuberculosis* specific for seven distinct antigens were used to screen for cross reactivity with the secretory fractions of *S. albus.* Of the seven antibodies, only one gave a positive hit. This is a monoclonal antibody directed at a specific internal amino acid sequence in the secreted acid phosphatase of mycobacteria (SapM). The anti-SapM antibody reacted with two bands of approximately 30 kDa and 21kDa size in supernatant fractions but none of the cytoplasmic extracts reacted with this antibody which is almost the same sizes that we got in this study.

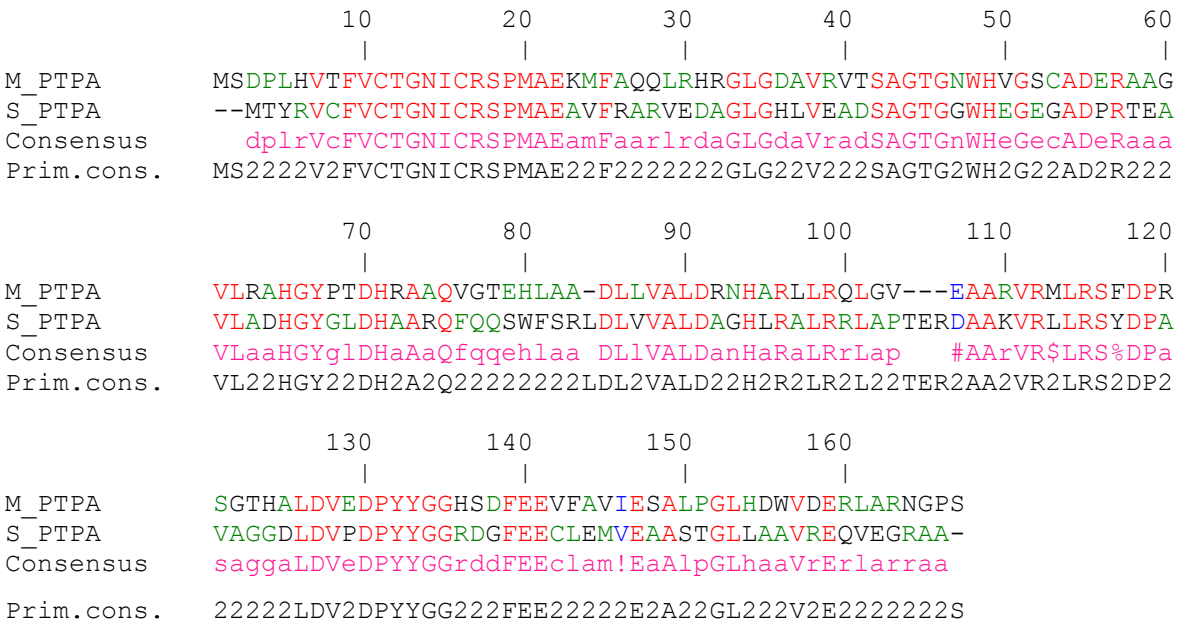

Figure 22. Comparison of the deduced amino acid sequence of *S. coelicolor* protein tyrosine phosphatase (S_PTPA) with amino acid sequence of *M. tuberculosis* (M_PTPA). Alignment data: Alignment length 163. Identities 50%. Positives 60%.

5. Conclusion

The clinical isolate of *S. albus* partially characterized in this study clearly exhibits some physiological attributes similar to those of its phylogenetic relative *M. tuberculosis*. It utilizes similar metabolic pathways as seen by the level of acidification of the Sauton's media when certain nitrogen sources are used in the media. The activity of acid phosphatases in the culture supernatant of *S. albus* appears to be regulated by levels of inorganic phosphate rather than by acid pH.

This pathogenic *S. albus* elaborates significant levels of acid phosphatase activity when cultured in Sauton's media; a minimal medium originally developed for growth of Mycobacteria. Although SapM of Mycobacteria shows sequence homologies to a protein of similar size from various Streptomyces, acid phosphatase activity in the culture supernatant of *S. albus* appears to be due to the presence of other phosphatases unrelated to SapM.

Elaboration of significant acid phosphatase activity in culture further supports the proposition of physiological relatedness of *S. albus* and *M. tuberculosis.* It also indicates that *S. albus* produces potential virulence factors that have been established in other intracellular pathogens as playing an important role in pathogenesis. These observations further support the idea of pathogenic Streptomyces as being good candidates as heterologous vaccine vehicles against Tuberculosis.

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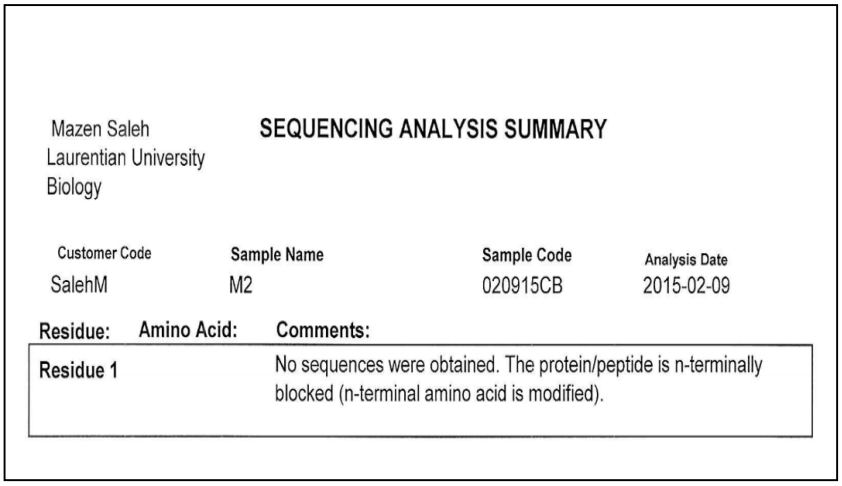
Appendix



Mazen Saleh
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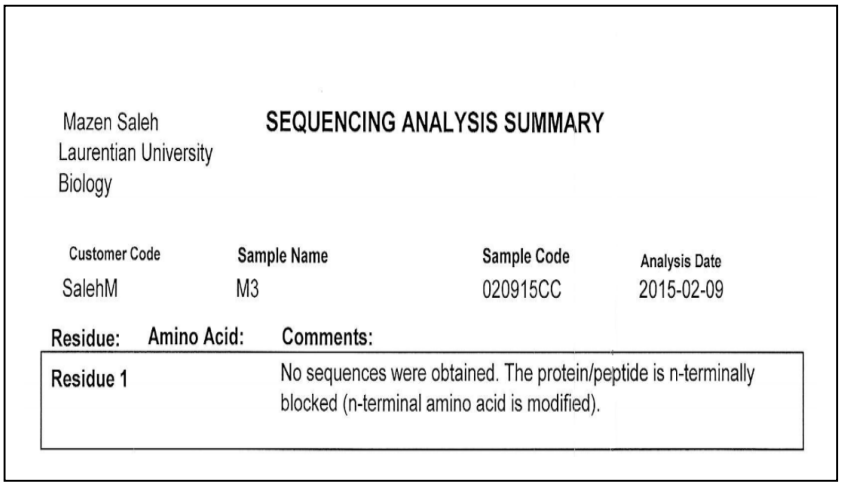
Biology

SEQUENCING ANALYSIS SUMMARY

Customer Code	Sample Name	Sample Code	Analysis Date
SalehM	M2	020915CB	2015-02-09

Residue:	Amino Acid:	Comments:
Residue 1		No sequences were obtained. The protein/peptide is n-terminally blocked (n-terminal amino acid is modified).



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SEQUENCING ANALYSIS SUMMARY

Customer Code	Sample Name	Sample Code	Analysis Date
SalehM	M3	020915CC	2015-02-09

Residue:	Amino Acid:	Comments:
Residue 1		No sequences were obtained. The protein/peptide is n-terminally blocked (n-terminal amino acid is modified).

Appendix 1: Sequencing analysis report indicating the blocked N- terminal of the purified proteins.