OBTAINING DURABLE ENZYME POWDER VIA SPRAY DRYING

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ABSTRACT

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Serine alkaline protease (SAP, EC 3.4.21.62) produced by *Bacillus* species, that are the microbioreactors within the bioreactors, is one of the major industrial enzymes. In this study, after production by Recombinant *Bacillus subtilis* (BGSC-1A751), carrying pHV1431*::subc* gene in the complex media and separation of solids, SAP was dried by using a spray drier. Experiments were performed to investigate the stabilization of SAP during spray drying and subsequent storage. Initially, the effect of air inlet temperature of the spray drier on SAP activity was evaluated. For this purpose, SAP solutions were spray dried in the absence of any protective agents at five different air inlet temperature (70^0C, 90^0C, 110^0C, 120^0C, 130^0C). As a result, increasing air inlet temperature lead to an increase in activity loss of SAP during drying. Thereafter, the effect of protective additives, glucose and maltodextrin (0.5%, 1%, 2% w/v), on SAP activity was investigated during spray drying. The activity loss of SAP was completely inhibited in the presence of glucose at 70, 90 and 110^0C. However, the addition of maltodextrin was better than glucose for activity preservation of

SAP at 130^{0}C. Among the obtained results, 1% glucose addition was the best to preserve activity of SAP during spray drying. Then, structural change of SAP during drying was investigated. FTIR-ATR spectrum was used to evaluate the change in physical structure of the dried SAP powders in the presence of 1% glucose. From infrared images, at 90^{0}C more native-like structure for dried SAP powders was observed. In the last stage of this study storage stability of obtained SAP powders at 4^{0}C for a long period (6 months) was investigated. When considering all conditions, 0.5% maltodextrin addition was the best for stabilizing SAP powders along storage time.

Key Words: Spray drying; Serine Alkaline Protease; Protective Additives; Activity Loss; Storage Stability

ÖZ

SPREY KURUTMA YÖNTEMİ İLE KALICI ENZİM TOZU ELDE EDİLMESİ

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Biyoreaktör içinde mikrobiyoreaktör olarak işlev yapan *Bacillus* türü mikroorganizmalar tarafından üretilen serin alkali proteaz (SAP, E.C. 3.4.21.62) endüstriyel enzimlerin en önemli gruplarından biridir. Bu çalışmada, pHV1431*::subc* geni taşıyan rekombinant *Bacillus subtilis* (BGSC-1A751) ile kompleks ortamda üretimden ve katıların ayrılmasından sonra SAP enzimi sprey kurutucu kullanılarak kurutulmuştur. Deneyler, kurutma ve sonrasında gelen depolama sırasında SAP'ın aktivitesini incelemek için yapılmıştır. Öncelikle, sprey kurutucunun hava giriş sıcaklığının SAP aktivitesine etkisi değerlendirilmiştir. Bu amaçla, SAP çözeltileri koruyucu ajanların yokluğunda beş farklı hava giriş sıcaklığında (70^0C, 90^0C, 110^0C, 120^0C, 130^0C) sprey kurutulmuştur. Sonuçta, hava giriş sıcaklığının artması kurutma boyunca SAP aktivite kaybının artmasına neden olmuştur. Sonra, koruyucu katkı maddelerinin, glikoz ve maltodextrin (%0.5, %1, %2 ağırlık/hacim), kurutma boyunca SAP aktivitisine etkisi

araştırılmıştır. Glikoz varlığında 70,90 ve 110^0C'de SAP aktivite kaybı tamamen önlenmiştir. Bununla birlikte, SAP'ın 130^0C'de aktivite koruması için maltodextrinin eklenmesinin glikoza gore daha olumlu sonuçlar verdiği görülmüştür. Elde edilen sonuçlar ışığında, sprey kurutmada %1 glikoz eklenmesinin SAP aktivitesinin korunmasını en yüksek oranda sağladığı söylenebilir. Daha sonra, kurutma boyunca SAP'ın yapısal değişikliği araştırılmıştır. FTIR-ATR spektrumu kullanılarak %1 glikoz varlığında kurutulmuş SAP tozlarının fiziksel yapısındaki değişiklik değerlendirilmiştir. Bu analizlerden, doğal yapısına daha çok benzeyen SAP tozlarının 90^0C'de olduğu gözlemlenmiştir. Bu çalışmanın son basamağında, elde edilen SAP tozlarının uzun bir süre (6 ay) için 4^0C'deki saklama kararlılığı araştırılmıştır. Bütün çalışma koşulları göz önüne alındığında, saklama süresi boyunca %0.5 maltodextrin eklenmesi SAP tozlarının kararlılığı için en uygunudur.

Anahtar Kelimeler: Sprey kurutma; Serin Alkali Proteaz; Koruyucu katkı maddeleri; Aktivite Kaybı; Saklama kararlılığı

To My Dad, Mom and Sister

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NOMENCLATURE

A	SAP activity, U cm^{-3}
A_λ	Absorbance
C_G^0	Initial glucose concentration, kg m^{-3}
$C_{soybean}^0$	Initial soybean concentration, kg m^{-3}
$C_{sucrose}^0$	Initial sucrose concentration, kg m^{-3}
C_X	Cell concentration, kg dry cell m^{-3}
N	Agitation rate, min^{-1}
PH_0	Initial pH
Q_o	Volumetric air feed rate, m^3 min^{-1}
T	Temperature, °C
U	One unit of an enzyme
v	Volume, ml
V	Volume of the bioreactor, m^3
V_R	Volume of the bioreaction medium, m^3
w	Weight, g

Greek Letters

λ	Wavelength, nm

Abbreviations

ATP	Adenosinetriphosphate
CCE	Crude cellular extract
CMC	Carboxymethylcellulases
CoA	Coenzyme A
DE	Dextrose Equivalent
DFP	Diisopropylfluorophophate
EC	Enzyme Commission

FAD	Flavine adeninedinucleotide
FDA	American Food and Drug Administration
FTIR-ATR	Fourier transform infrared spectroscopy with attenuated total structure reflectance
HGH	Human growth hormon
GRAS	Generally recognized as safe
MD	Maltodextrin
MW	Molecular weight, g/gmol
PEG	Polyethyleneglycol
PMSF	Phenylmethylsulponylflouoride
PVP	Poly(vinyl)pyrrolidone
SAP	Serine alkaline protease
SD	Spray drier
T	Residence time, h
t-PA	Tissue type plasminogen activator
TCA	Tricarboxylic acid

CHAPTER 1

INTRODUCTION

Drying is usually the final step in a series of operations, and the product from a dryer is often ready for final packaging. The reasons for drying are almost as diverse as the materials that are dried. Sometimes drying is carried out to affect economics in freight or to ease handling. Many materials must be dried to bring their moisture content to a prescribed value before being sold. Others foodstuffs, biological materials and pharmaceuticals are dried to preserve them for storage and shipment without the need for refrigeration (Keey, 1978).

Enzymes are one of the most important groups of biotechnological products and they serve important functions in detergent, food, pharmaceutical and biochemical processes (Kalizs,1988). In order to increase the shelf life, ease storage and transport, reduce transportation cost and protect the biological activity of these molecules they are often preserved in dry form. Carbohydrates and polymers are often used as bulking agents and protective additives in this context.

There are number of conventional drying techniques used in the production of variety of goods. These techniques include freeze drying, air drying and spray drying. Freeze drying is the most common technique for obtaining a dry

formulation of enzymes. As operated on the principle of ice sublimation, it is usually considered to have less thermal effect on the enzyme when compared to other drying methods. However, the process has several disadvantages; during the freezing step, enzyme and buffer components tend to be concentrated in the phase between ice crystals. Such a concentration effect can result in a dramatic change in the pH and ionic strength of the enzyme environment and thus, lead to a change in biological activity, *i.e.*, denaturation. To reduce the degree of denaturation, stabilizing agents are added to a protein solution prior to freeze drying. Tankana et *al.* (1991) studied the mechanism of the cryoprotective effect of saccharides on the denaturation of catalase in the freeze-drying process. They observed that saccharides with longer glucoside chains have less protection effect compared on the basis of their weights and saccharides are bound to catalase as monomolecular layer and this layer protects catalase from being denaturated instead of the hydration monolayer. Subsequent study was performed by Chang et *al.* (1996) and they found that the denaturation of proteins during freezing is closely related to surface-induced denaturation. They stated that nonsurfactant additives, which are also potent stabilizers (e.g., sugars and polymers) increase the overall thermodynamic stability of the native protein, which is at least weakly associated with resistance to denaturation at surfaces. Later on Millqvist-Fureby et. *al.* (1999b) investigated the surface composition of freeze-dried protein/carbohydrate mixtures and related the findings to the properties of the carbohydrates and to the residual activity of a model enzyme (trypsin) after drying. This study showed that proteins indeed accumulate at the surface of the freeze-dried protein/carbohydrate powders. The degree of surface accumulation depended on the carbohydrate used and the activity of trypsin was fairly well preserved in the freeze-dried powders, depended on the carbohydrate excipient, the surface composition had little effect on the activity.

When compared with other drying processes, freeze drying is an energy-intensive and time-consuming process also raises the concern of high production cost (Belghith *et al.,* 2001; Mumenthaler *et al.*, 1994).

Alternative techniques for preparing dry forms of proteins have been reported in the literature. Allison et *al.* in 1998 compared vacuum drying and freeze drying as dehydration methods and discerned the relative capacities of sucrose, dextran, and sucrose/dextran mixtures to protect both structure and function of a model protein, rabbit skeletal muscle actin, during these processes. They resulted that with either drying process, the most common way in which recovery of native protein has been increased is to include stabilizing additives in the initial protein solution.

Among the conventional drying processes that rely on evaporation, spray-drying is currently a well-established method for processing liquids into powders. Unlike freeze drying, spray drying-utilizes heat from a hot gas stream to evaporate micro-dispersed droplets created by atomization of a continuos liquid feed and is therefore a very fast and cost-effective dehydration method.

Some spray dried enzymes and their applications in industry are given in Table1.1 (Masters, 1985).

Serine alkaline protease, which is the protein used in this study, is one of the most important groups of industrial enzymes since it accounts for approximately 35% of the microbial enzyme sales. The major application of serine alkaline protease is in detergent industry (Kalizs, 1988).

Table 1.1 Uses of spray-dried enzymes

Enzyme	Use
Amylase	Baking, milling, paper, textiles, brewing
Protease	Brewing, meat fish tenderizing, detergents, cheese making, leather tanning, oral hygiene
Glucose oxidase	Carbonated beverages
Pectinase	Coffee fermentation, juice clarification
Lipase	Detergents, digestive aids
Trypsin	Wound debridement
Rennin	Cheese making
Lactase	Ice cream
Cellulase	Cellulose breakdown

In literature, there have been various publications related with spray drying of enzymes and other proteins. Yamamoto and Sano (1991) measured the average moisture content, drop temperature and the relative remaining enzyme activity as a function of drying time during drying of a single suspended droplet (d_p = 2mm) of sugar solutions containing β-galactosidase, glucose oxidase or alkaline phosphate. They resulted that the lower temperature and small droplets give a higher enzyme retention while the effect of the initial water content is weak and the choice of the dissolved solid is quite important for obtaining a high enzyme retention. Broadhead *et al.* (1994) studied the effects of process variables (temperature, solution feed rate and air flow rate), formulation parameters (total solids level, ratio of stabilizer to protein, presence of surfactant

and buffer) on the characteristics of a model spray-dried protein β-galactosidase and the effects of stabilizers (mannitol, sucrose, arginine hydrochloride and trehalose) in terms of their ability to preserve enzymatic activity during the spray-drying process and during long-term storage. They obtained highest product yields and extensive protein denaturation at high drier outlet temperature and they found that trehalose was the most suitable stabilizer and spray-drying operates best when the feed solution has a higher solid content. Fäldt and Bergenstahl (1994) investigated the surface composition of the spray dried lactose and protein mixtures and the structures of these powders. Their results indicated that even with a low concentration of protein (0.01 wt.%) in the solution to be dried, protein starts to be appear on the surface of the powder and when the surface coverage of protein increases, dents start to appear in the particles. Mumenthaler *et al.* (1994) investigated the spray drying of re-combinant methionyl human growth hormone (hGH) and tissue-type plasminogen activator (t-PA). They focused on preparing spray-dried protein powders, which upon reconstitution, possessed a quality comparable to the liquid bulk. They found that spray drying of mannitol formulated human growth hormone results in extensive protein aggregation, which are formed primarily by surface-induced denaturation of the protein at the air-liquid interface during atomization. Millqvist-Fureby *et al.* (1999) investigated the surface accumulation, activity loss of trypsin (protein) and the effect of carbohydrates and surfactants during drying. According to their results, protein/carbohydrate mixtures show a strong protein accumulation, which is due to protein adsorbing to the air/water interface of the spray droplets prior to solvent evaporation, at the powder surface, the activity of model protein trypsin was well preserved with all carbohydrates, and addition of the surfactant the surface accumulation of protein was reduced and the residual activity was increased. Belghith *et al.* (2001) studied thermal stability at various air inlet and outlet temperatures of

the spray dryer, the effect of some additives (PEG and MD) on the spray drying of cellulases and the stability of such atomised enzymes during storage at 4^0C and 30^0C for up to eight months. They found best enzyme recovery at low inlet-outlet temperature in the absence of additives and they observed that 1% MD stabilized cellulases even after a long period of storage at 30^0C and 1% PEG enabled the best enzyme recovery. Depaz *et al.* (2002) examined the effects of disaccharides (sucrose and trehalose), polymers (dextran and maltodextrin) and disaccharide-polymer mixtures on the stability of subtilisin, a common industrial laundry detergent enzyme, during drying and during subsequent storage in dried solids containing perborate, a laundry detergent additive that hydrolyzes to form hydrogen peroxide in the presence of water. Their results showed that dissaccharides, which can hydrogen bond to subtilisin in the place of lost of water, inhibit dehydration-induced unfolding of subtilisin, however none of the additives protected Met222 residue in enzyme subtilisin from oxidation during storage of the initially protein formulations at 30^0C and 20 or 75% relative humidity under oxidizing conditions. Vasiljevic and Jelen (2003) studied to explore the consequences of spray and freeze drying on the β-galactosidase activity from *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 in dried crude cellular extract (CCE) preparations. They emphasized on the effectiveness of drying adjuncts of dairy origin on the enzyme activity preservation, and the effect of storage on the stability of the β-gal activity. They resulted that the addition of dairy-based drying adjuncts to CCE preparations had a major effect on the preservation of the β-gal activity during both drying process and during storage and increasing the adjunct concentration improved the retention of the enzyme activity.

The aim of this study is to obtain stable spray-dried serine alkaline protease (SAP) powders with minimum activity loss. In this context, effect of temperature and additives (glucose, maltodextrin) on the activity of SAP during drying and the structures of the obtained powders were investigated. Thereafter, the stability of SAP powders during storage at 4^0C was investigated.

CHAPTER 2

ENZYMES AND THEIR BIOTECHNOLOGICAL PROCESS

2.1 Enzymes

2.1.1 General Characteristics

Enzymes, which are proteins in nature, are catalyst of biological systems. The main functional characteristics of enzymes compared with chemical catalysts are their high efficiency and specificity. They accelerate the rate of the reaction toward equilibrium without changing the position of that equilibrium. Most of the reactions they catalyze would not proceed in their absence in a reasonable time without extremes of temperature, pressure or pH (Moran, 1994).

Enzymes are characterized by high substrate and functional specificities. The substrate specificity means that the enzyme catalyzes the reaction of only one chemical compound or a group of chemically very similar compounds. The functional specificity is expressed by the fact that an enzyme catalyses only one definite reaction and the substrate undergoes a definite conversion (Präve, 1987).

Another distinguishing characteristic of enzymes is their frequent need for cofactors. A cofactor is a non-protein compound which combines with an other-

wise inactive protein to give a catalytically active complex. The simplest cofactors are metal ions like Ca^{2+}, Zn^{2+}, Co^{2+}, etc. On the other hand, a complex organic molecule called a coenzyme may serve as a cofactor such as NAD, FAD, CoA, or some vitamins (Bailey, 1986).

2.1.2 Classification of Enzymes

Most enzymes are named by adding the suffix *–ase* to the name of the substrate they act upon or to a descriptive term for the reactions they catalyze (Moran, 1994). In accordance with the types of reactions which they catalyze, enzymes are classified in six main classes. Each of the major classes is further divided into numerical sub-classes according to the individual reactions and the nature of substrates involved (Atkinson and Mavituna, 1991). Each enzyme is then assigned an EC (enzyme commission) four-digit classification number and a systematic name, which identifies the reaction catalyzed. For example, the EC number of serine alkaline protease is EC 3.4.21.14. Table 2.1 summarizes the classification of enzymes.

2.1.3 Enzyme Activity

In the formation of substrate-enzyme complex, the substrate binds to a specific region on the enzyme called active site, where reaction occurs and products are released (Bailey, 1986). The binding ability depends on the structure of the protein and is lost on denaturation. An enzyme is characterized by its catalytic activity, which is usually given as a measure of the amount enzyme (Präve, 1987). The Commission on Enzymes suggested that a standard unit definition of enzyme activity should be as:

One unit (U) of any enzyme is defined as that amount which will catalyze the transformation of one micromole of substrate per minute under defined conditions.

Table 2.1 International classification of enzymes.

No	Class	Type of reaction catalyzed
1	Oxidoreductases	Transfer of electrons
2	Transferases	Group-transfer reactions
3	Hydrolases	Transfer of functional groups to water
4	Lyases	Addition of groups to double bonds or the reverse
5	Isomerases	Transfer of groups within molecules to yield isomeric forms
6	Ligases	Formation of C-C, C-S, C-O, and C-N bonds by condensation reactions coupled to ATP cleavage

2.1.4 Influences on Enzyme Activity

All enzymes are proteins and the protein secondary structure is stabilized by weak forces, often giving rise to functionally important molecular flexibility. On the other hand, this weak stabilization implies that proteins are less biologically active configurations. Many factors can influence the catalytic activity of enzymes, presumably by affecting the enzyme's structural or chemical state. Included among these factors are (Bailey, 1989):

1. pH

2. Temperature

3. Fluid forces (hydrodynamic forces, hydrostatic pressure and interfacial tension)

4. Chemical agents (such as alcohol, urea and hydrogen peroxide)

5. Irradiation (light, sound, ionizing rate)

2.2 Proteolytic Enzymes

Proteolytic enzymes hydrolysing peptide bonds are in fourth sub-group of the third basic group (hydrolyses) of enzyme classification. Microbial proteases are classified into two major groups on the basis of their nature of attack, peptidases and proteases. The exopeptidases remove terminal amino acids or dipeptides and are of the secondary importance since they cannot rapidly complete digestion. Proteases are involved mainly in degradation of very large polypeptide substrates into peptides and amino acids before cellular uptake. Proteases are classified by their catalytic mechanism into four groups. These are (Kalisz, 1988);

1. Serine Proteases (3.4.21)

2. Cysteine Proteases (3.4.22)

3. Aspartic Proteases(3.4.23)

4. Metalloproteases (3.4.24)

2.2.1 Serine Proteases

The serine proteases are the most widely distributed group of proteolytic enzymes of both microbial and animal origin. The enzymes have reactive serine

residue in the active site and are generally inhibited by either DFP or PMSF. Many are also inhibited some thiol reagents probably due to the presence of a cysteine residue near the active site which probably does not participate in the catalytic mechanism of the enzyme. Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7-11. They have low molecular weights (18.5-35 kDa). Most have isoelectric points between pI 4.4 and 6.2 (Çalık et *al*., 2001).

Serine proteases can be divided into four sub-groups, according to their side chain specificity. These are (Kalizs, 1998);

1. Trypsin-Like Proteases

2. Alkaline Proteases

3. Myxobacter α-Lytic Proteases

4. Staphylococcal Proteases

2.2.1.1 Serine Alkaline Proteases (SAP)

Serine Alkaline Proteases (SAP) is one of the most important groups of industrial enzymes that are widely used in detergent, leather and meat industries. They are produced by various bacteria, moulds and yeasts (Kalisz, 1998). They account for approximately 35% of the microbial enzyme sales. The common properties of all serine alkaline proteases are (Çalık et *al*., 2001):

1. They all involve a particular serine residue that is essential for their catalytic activity; and

2. They are most active at approximately pH=10.

The amino acid sequence of these enzymes depends on the microorganism that they are produced by. However, whatever their amino acid composition is, they fold in such a way that histidine, aspartic acid and serine form a catalytic triad. Near the active site is a hydrophobic binding site, a slit-like pocket that preferably accommodates the non-polar side chains; thus serine alkaline proteases are specific for aromatic and hydrophobic residues such as tyrosine, phenylalanine, tryptophan and leucine. They are most active at around pH 10 and their molecular weights are in the 15-30 kDa range. Their optimum temperature is 70^0C and The isoelectric point of SAP is normally around pH 9 (Çalık et *al*., 2001).

2.3 Production of Enzymes

2.3.1 Sources of Enzymes

Enzymes are obtained from animal, plant, and microbial starting material. In the last 30 years, microorganisms have achieved a paramount importance, but enzymes from animal and plant material are nevertheless still important for special applications (Präve, 1987).

In industrial production of enzymes, microorganisms are mainly utilized for the following basic reasons:

1. Enzyme levels can be increased by environmental and genetic manipulations;
2. Enzyme fermentations are quite economical on a large scale;
3. Screening procedures are simple (Fogarty and Kelly, 1990).

2.3.2 Selection of Suitable Microorganism

The selection of host microorganism for production of industrial enzymes is critical for the commercial success of the product. Potential hosts should give sufficient yields, be able to secrete large amounts of protein, be suitable for industrial fermentations, produce a large cell mass per volume quickly and on cheap media, be considered safe based on historical experience or evaluation by regulatory authorities, and should not produce harmful substances or any other undesirable products (Kirk and Othmer, 1994). Traditionally, identification of the most suitable enzyme source involves screening a wide range of candidate microorganisms.

Serine alkaline proteases are produced by various species of bacteria, moulds and yeasts. Among many species, *Bacillus* strains which fulfil all of the aforementioned criteria have the capacity to secrete large quantities of extracellular enzymes and this makes the genus *Bacillus* more favourable than the others for protease production from the aspects of yield, selectivity, and productivity. In the literature, Hanlon and Hodges (1981), Frankena et *al.* (1985, 1986), van Putten et *al.* (1996), Hübner et al (1993) and Çalık et *al.* (1998,2000) used *B. licheniformis*; Kole et *al.* (1998) and Çalık et *al.* (2003a, b) used *B.subtilis*, Moon and Parulekar (1991,1993) used *B.firmus*; Wright et *al.* (1992) used *B.brevis* for the SAP production.

2.3.2.1 The Genus *Bacillus*

The rod-shaped bacteria that aerobically form endospores are assigned to the genus *Bacillus*. The endospores of the bacilli are resistant to heat, drying, disinfectants and other destructive agents, and thus may remain viable for centuries.

The genus *Bacillus* encompasses a great diversity of strains. Specifically, *B.subtilis* assigned to Group 2, produce oval endospores that do not swell the mother cell. They are gram positive, are motile by peritrichous flagella, and produce acids from a range of sugars. They are listed by the American Food and Drug Administration (FDA) as a GRAS (Generally recognized as Safe) organism. *B.subtilis*, generally regarded as an aerobe, has pH and temperature tolerance in the range of 5.4-8.0 and 20°C-55°C respectively (Priest, 1993; and Laskin and Lechevalier, 1973).

2.3.3 Production Media

The production media must contain sources of carbon and energy, and also of nitrogen, together with the necessary mineral substances. On the other hand, the medium should not contain substances that cause the repression of the enzyme production (Präve, 1987).

There are two major types of media, which are defined and complex media. Defined media contain specific amounts of pure chemical compounds with known chemical compositions. Complex media contain natural compounds whose chemical composition is not exactly known. A medium containing soybean, yeast extract, peptone, molasses, or corn steep liquor is a complex medium. A complex medium usually can provide the necessary growth factors, vitamins, hormones, and trace elements, often resulting in higher cell yields, compared to the defined medium. Often complex media are less expensive than defined media. Among the production media, complex media are used in the industrial bioprocesses since, the attainable enzyme activity and cell yields are much higher than that of the defined media (Özdemir, 2003).

Çalık et al., (2003) investigated the effects of complex medium components, i.e. carbon sources (glucose, sucrose, molasses, and defatted-

soybean), nitrogen sources ($(NH_4)_2HPO_4$, casein and defatted-soybean) and inorganic compounds on SAP production by recombinant *Bacillus subtilis* carrying pHV1431::*subC* gene. They reported that maximum activities were obtained as A = 3050 cm^{-3} with $C^0_{soybean}$ = 20 kg m^{-3} and C^0_G = 8 kg m^{-3}, and as A = 3850 cm^{-3} with $C^0_{soybean}$ = 20 kg m^{-3} and $C^0_{sucrose}$ = 15 kg m^{-3}; further increase in production was obtained by optimizing the phosphate ion concentration, i.e. A = 5350 cm^{-3} , in the medium containing $C^0_{soybean}$ = 20 kg m^{-3} and $C^0_{sucrose}$ = 15 kg m^{-3}.

2.3.4 Fermentation

In fermentation processes, raw material (biological or non-biological) is transformed into some product by means of microorganisms, animal or plant cell cultures, or by cell materials (e.g. enzymes, organelles) (Moses and Cape, 1991).

The cells continuously strive by modifying their environment to achieve and maintain the optimal conditions for their growth. In a bioreactor, this tendency of the cells is assisted by providing nutrition and suitable condition. The reactor has task of ensuring the supplying of the cells with the means for growth or for the production of metabolites, i.e., of guaranteeing as far as possible the optima of the temperature and pH and a sufficient supply of substrate, nutrient salts, vitamins, and oxygen.

The optimum conditions for the selected strains must be determined experimentally. This is carried out in the laboratory, frequently in the shake cultures. However, these have the disadvantage that their pH value and the concentration of the dissolved oxygen cannot, as a rule, be controlled. Consequently, only the optimum temperature and the composition of the

nutrient solution and the supplementation of the substrate in them can be determined. The optimization of the pH and of the concentration of the dissolved oxygen is generally carried out in small laboratory reactors which should be provided with a pH control and, if possible, with stirrer speed and gas flow measurement. Enzyme production usually takes place in mechanically stirred tanks in batch operation (Präve, 1987).

2.4 Separation of Solids

Solid-liquid separation is a central basic operation in the isolation of enzymes. This process step is necessary both in the separation of the cells from the culture broth and also in the clarification crude extract after disintegration of the cells and the elimination of the cell fragments, in the separation of precipitates after precipitation operation, and, sometimes, in the separation of added absorbents from protein-containing solutions. Two procedures are available for this task: centrifugation and filtration. Filtrations can serve two purposes: either the recovery of the precipitate, or the separation of the undissolved components from a process solution (Präve, 1987).

2.4.1 Centrifugation

The tubular-bowl centrifuges that are important for the separation of cell constituents possess clarification cylinders with relatively small diameters (5 to 15 cm), which permits high velocities in rotation. As a rule, the liquid is fed in at the bottom through a hollow shaft. The machine is driven from the top via belts (up to 20 000 rpm) or, in a special form, by turbines (up to 55 000 rpm). The sediment is thrown against the wall of the rotor, while the liquid rises to the top (Präve, 1987).

2.5 Drying

The word drying is used to describe any process in which volatile substance is removed by heat from a mixture that yields a solid product. Commonly, the principal volatile substance is water and in drying, the water is usually removed as vapor by air.

Drying processes can also be categorized according to the physical conditions used to add heat and remove water vapor: (1) in the first category, heat is added by direct contact with heated air at atmospheric pressure, and the water vapor formed is removed by the air; (2) in vacuum drying, the evaporation of water proceeds more rapidly at low pressures, and the heat is added indirectly by contact with a metal wall or by radiation; and (3) in freeze drying, water is sublimed from the frozen material (Geankoplis, 1993).

Drying is an important unit operation in the biotechnological process of enzymes. By this technique, enzymes which are unstable in their aqueous solutions, are preserved in their dry form. However, the removal of water reduces the movement freedom of the enzyme molecules and thus inhibits conformational changes leading to activity loss. To eliminate this activity loss during drying the appropriate additives may be chosen. Protective additives help to maintain protein structure during drying via hydrogen bonding (sucrose, glucose, lactose, and trehalose) and form an amorphous phase (sucrose, trehalose, dextran, glucose, lactose, and maltodextrin) (Millqvist-fureby et *al*., Depaz et *al*.). Drying processes that may be considered to obtain stable enzyme powders are freeze-drying, vacuum-drying, and spray-drying.

2.5.1 Selection of Drying Equipment

The first consideration in selecting a dryer is its operability; above all else, the equipment must produce the desired product in the desired form at the desired rate. And also the selected dryer must operate reliably, safely, and economically (Geankoplis, 1993). As mentioned before, enzymes must be dried at low temperature under vacuum, with a low-temperature heating medium, or very rapidly as in spray dryer. Among these dryers, spray dryer is very attractive because of its lower cost and a solid powder can be obtained from a liquid feed in a very short period by using this process.

2.5.2 Spray Drying

Spray drying is a unique drying process since it involves both particle formation and drying. The characteristics of the resultant powder can be controlled, and powder properties can be maintained constant throughout a continuous operation (Masters, 1985)

In a spray dryer a slurry or liquid solution is dispersed into a stream of hot gas in the form of a mist of fine droplets. Moisture is rapidly vaporized from the droplets, leaving residual particles of dry solid, which are then separated from the gas stream.

2.5.2.1 Basic Components of a Spray Dryer

Spray drying consists of four process stages :

1. Atomization of feed into a spray

2. Spray-air contact (mixing and flow)

3. Drying of spray (moisture/volatile evaporation)

4. Separation of dried product from the air

A typical spray dryer is shown in Figure 2.5.2 (Geankoplis, 1993).

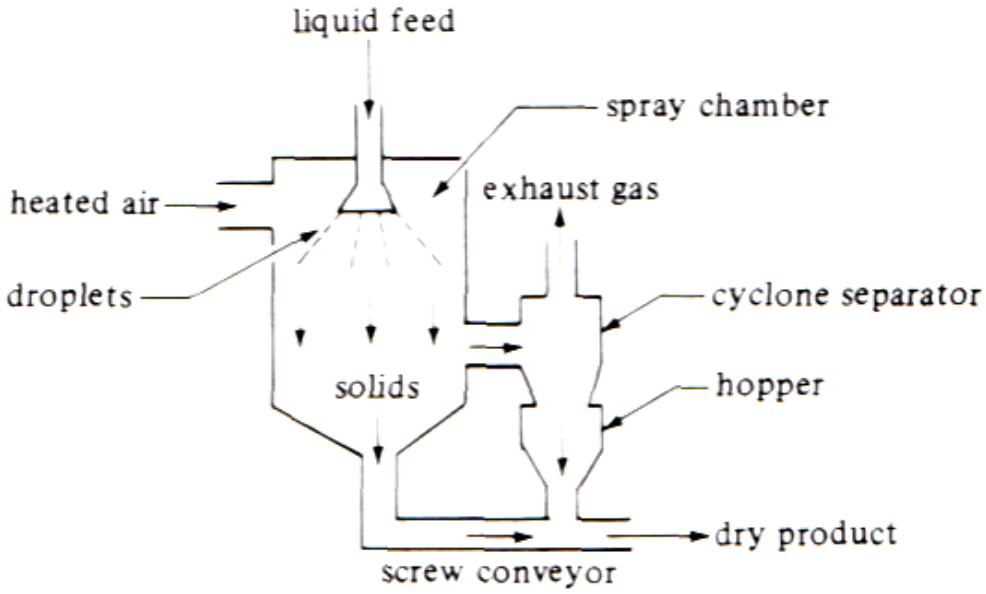

Figure 2.5.2 Spray dryer

2.5.2.2 Effect of Operating Variables on Dried-product

Feed Properties

Increase in feed viscosity through increase in feed solids or reduction in feed temperature will produce coarser sprays on atomization at fixed atomizer

operating conditions. Surface-tension effects appear minor. Increase in feed solids affect evaporation characteristics where generally an increase in particle and bulk density results.

Feed Rate

Increasing feed rate at constant atomizer operating conditions produces coarser sprays and dried products.

Air Flow

The rate of air flow controls to a certain extent the residence time of the product in the drying chamber. Increased residence time leads to a greater degree of moisture removal. Reducing air velocity assists product recovery from the drying chamber.

Drying Temperatures

Inlet

Increase in inlet temperature increases the dryer evaporative capacity at constant air rate. Higher inlet temperatures mean a more thermally efficient dryer operation. Increased temperature often causes a reduction in bulk density, as evaporation rates are faster, and products dry to more porous or fragmented structure.

Outlet

For a fixed moisture content and dryer design, outlet temperature must be kept within a narrow range to maintain the powder packing and flow requirements. Increase in outlet temperature decreases moisture content at

constant air flow and heat input conditions. Operation at low outlet temperature to produce powder of high moisture content is used when agglomerated forms of powder are required. This is often utilized in making special 'instant' powders (Masters, 1985).

2.5.2.3 Advantages of Spray Drying

1. Powders are produced of specific particle size and moisture content irrespective of dryer capacity and product heat sensitivity.

2. The specification or powder quality remains constant throughout the entire dryer operation irrespective of the length of the dryer run once conditions are held constant.

3. Spray dryer operation is continuous and easy, operation is adaptable for full automatic control, response times are fast.

4. A wide range of dryer designs are available. Product specifications can be easily met.

5. Spray drying is applicable to both heat sensitive and heat resistant materials.

The main drawbacks of spray drying are high installation cost of the equipment and poor thermal efficiency (Masters, 1985).

2.5.2.4 Literature Review on Spray Drying of Enzymes and Proteins

Spray drying is used extensively for drying of heat sensitive materials including enzymes, blood products and microorganisms. In spite of the high temperatures which are involved, the cooling effect caused by solvent evaporation leads to relatively low temperature of the dried product (Masters, 1985). Therefore, biologically active materials such as enzymes can be spray-

dried without appreciable activity losses. But, drying in general may also alter the secondary structure of the protein, since the hydration of the protein is partly lost. By replacing water with excipients, e.g. sucrose and lactose which are capable of forming hydrogen bonds with the protein, this effect can largely be compensated for. Other sources of loss of enzyme structure, and thus activity, are the shear forces in the spray nozzle, as well as enzyme adsorption at the droplet surface (Carpenter and Crowe,1989). Millqvist-Fureby *et al.* (1999) stated that minimizing the surface accumulation of the enzyme in the spray droplets should suppress the activity loss in the drying process. They made an investigation about surface accumulation and activity loss of a model protein (trypsin) during spray drying. They observed that the level of the protein accumulation at the powder surface can be controlled by adding a non-ionic surfactant and they observed well activity preservation with all carbohydrates.

The spray drying process is obviously a valuable alternative to freeze drying. Spray drying requires shorter processing time than freeze drying that is a multi-step and time-consuming process. Moreover it is less expensive and can be used at large scales (Belghith *et al.*, 2001).

Different parameters influence the quality of the powder obtained by spray drying: nature of the product, concentration in dry matter, inlet and outlet temperature of drying air, temperature in down vessel (air outlet) and flow rate of liquid injection. Belghith *et al.* (2001) examined the thermal stability and the effect of additives (PEG and maltodextrin) on the spray drying of cellulases, which are considered to become successfully commercialized in the future, and the stability of such atomised enzymes during storage at 4^0C and 30^0C. They showed that maltodextrin had a negative effect on enzyme recovery but it completely stabilized cellulases even after a long period (8 months) of storage at

30^0C, inversely PEG had a negative effect on enzyme stability but enabled the best recovery.

A common impediment to the production of the commercial proteins and enzymes is their marginal stability in aqueous solution. Water facilitates or mediates a variety of physical and/or chemical degradation pathways operative during protein purification, shipping, storage and/or delivery. Consequently, dry solid formulations are often developed to provide an acceptable protein shelf life. However, dehydration itself is a stress to proteins, causing protein unfolding, and if freeze drying is used, freezing is an additional source of stress. In order to extend the shelf lives of dried proteins and enzymes, the appropriate additives have been used. Depaz *et al.* (2002) focused on the effects of these additives (disaccharides (sucrose and trehalose), polymers (dextran and maltodextrin) and disaccharide-polymer mixtures) on the stability of subtilisin, a common industrial laundry detergent enzyme, during drying and during subsequent storage. They stated that additives capable of hydrogen bonding inhibit unfolding of subtilisin during drying.

CHAPTER 3

MATERIALS AND METHODS

3.1 Production of Serine Alkaline Proteases

3.1.1 Microorganism

Recombinant *Bacillus subtilis* (BGSC-1A751), carrying pHV1431*::subc* gene was used as a producer of serine alkaline protease (SAP, EC: 3.4.21.62).

3.1.2 Media

The microorganisms, stored in microbanks, were inoculated into the agar slants under sterile conditions and they were incubated at 30^0C for 48 h. The composition of solid medium for serine alkaline protease production by *Bacillus* sp. is given in Table 3.1 (Çalık, 1998).

Cells from slants were inoculated into pre-cultivation medium and microorganisms were grown at 37^0C and N=200 min^{-1} for 6 h. Laboratory batch experiments were conducted in orbital shakers under agitation and heating rate control, using air-filtered Erlenmeyer flasks 150 ml in size that had working volume capacities of 25 ml. The composition of the pre-cultivation medium for cell growth and enzyme production is provided in Table 3.2 (Çalık, 1998).

Table 3.1 The composition of solid medium for recombinant *Bacillus* sp.

Compound	**Concentration, kg m^{-3}**
$MnSO_4.2H_2O$	0.01
Agar	15.0
Peptone	2.5
Azocasein	2.0
Chloramphenicol	0.007

Table 3.2 The composition of precultivation medium for recombinant *Bacillus* sp.

Compound	**Concentration, kg m^{-3}**
Soytryptone	15.0
Peptone	5.0
$CaCl_2$	0.1
Na_2HPO_4	0.25
$MnSO_4.2H_2O$	0.01
Chloramphenicol	0.007

When the microorganism concentration was reached to 0.46 kg/m^{-3}, they were transferred either to the laboratory scale bioreactors (V=150 ml) or pilot scale bioreactor systems (V=2.0 dm^3) with a 1/10 inoculation ratio. The composition of the reference complex media is given in Table 3.3.

Table 3.3 The composition of the reference complex medium for recombinant *Bacillus* sp.

Compound	**Concentration, kg m^{-3}**
Defatted-soybean	20.0
Sucrose	15.0
Glucose	8.0
Na_2HPO_4	2.815
$NaH_2PO_4.2H_2O$	0.028
Chloramphenicol	0.007

Laboratory scale batch fermentation was conducted in agitation and heating rate controlled orbital shakers at N=200 min^{-1} and T=37 ^{0}C, using air filtered 150 cm^3 Erlenmeyer flasks that contained V_R=33 cm^3 fermentation mixture.

3.1.3 Bioreactor System Experiment

The pilot scale 2.0 dm^3 batch bioreactor systems (Braun CT2-2) consisted of a system of working volume 0.5-2.0x10^{-3} m^3 with temperature , pH, foam, stirring rate and dissolved oxygen controllers. It was stirred with two four-blade Rushton turbines. SAP fermentation was accomplished in working volume V_R=1.65 dm^3 and by using the agitation rate N=750 min^{-1} and the air rate Q_0/V_R = 0.5 vvm at T=37 ^{0}C and initial pH_0=7.1 (Çalık,2003c).

3.2 Drying

3.2.1 Materials

Glucose and maltodextrin, DE=13.0-17.0 (Sigma, [9050-36-6]) used as stabilizing agents in enzyme drying process and carboxymethylcellulose (CMC, Sigma, [9000-11-7]) used to prevent the dried particles to stick on the surface of drying chamber during drying. All these chemicals were used as received.

3.2.2 Spray Drying

The spray-drying runs were performed using a laboratory scale spray dryer (Lab-Plant Model SD-04). During operation, 100 ml prepared solution was fed at constant rate, 0.44 ml/min with a peristaltic pump to a nozzle, where atomization occurred by means of a pressurized air stream. Drying air entered the drying chamber in the same direction as the descending spray droplets (co-current operation). The process variable is the drier air inlet temperature (70, 90, 110, 120 and 130^0C). The outlet air temperature can not be controlled directly, but is a function of the drier inlet temperature and solution feed rate. According to the working inlet temperatures observed drier outlet temperatures was 56, 64, 76, 82 and 90^0C, respectively. In all of the experiments the feed solution rate was 0.44 ml/min and the air flow rate was constant. All drying experiments were repeated three times and their standard deviations were found as 6%.

3.3 Analysis

3.3.1 Cell Concentration of SAP

Cell concentrations based on dry weights were measured with a UV-Vis spectrophotometer (Thermo Spectronic, Heλios α) using a calibration curve (Appendix A) obtained at 600nm (Çalık, 1998).

3.3.2 Concentration of Prepared Solutions

After serine alkaline protease production, culture broth samples were centrifuged (Sigma 1-15) at 11,500 g for 15 minute at 4^0C and supernatant was separated. Stabilizing agents with varying concentrations 0.5%, 1%, 2% w/v (glucose and maltodextrin) were added to the obtained enzyme solutions (1.5% w/v dry weight) one by one. All starting preparations contained 0.04% w/v CMC.

3.3.3 SAP Activity

Activity loss of SAP was determined by measuring SAP activity before and after drying. The activity measurement of SAP after drying operation was accomplished by dissolving enzyme powder to obtain the same enzyme concentration as the starting enzyme solution before drying. For instance, before atomization: the concentration of 100 ml starting solution contains %1 glucose (%1.5 dry weight of enzyme solution + %1 glucose +%0.04 CMC) = (1.5x1)+(1x1)+(0.04x1) = 2.54 g / 100 ml. After atomization, the solution was prepared according to the same calculated concentration and then activity was measured.

After drying, storage stability of obtained SAP powders at 4^0C up to 6 months were investigated. Activity loss of SAP was determined by measuring SAP activity before and after storage. Again, activity measurement of SAP after storage was accomplished by dissolving enzyme powder to obtain the same enzyme concentration as the starting enzyme solution before drying.

Proteolytic activity was measured by the hydrolysis of casein. The culture broth was harvested by centrifugation at 13,500 g for 10 min at room temperature. Hammerstein casein (2 ml of 0.5% w/v) in borate buffer was mixed with 1 ml of diluted bacterial broth and hydrolyzed under T=37^0C, pH=10

and t=20 min. The reaction was stopped by adding 2 ml of 10% (w/v) trichloroacetic acid. After that the reaction mixture was centrifugated at 10,500 g for 10 min at $+4^0$C, and the absorbance of the supernatant was measured at 275 nm with a UV spectrophotometer (Appendix B). One unit protease activity was defined as the activity which liberates 4 nmole tyrosine min^{-1} (Çalık, 1998). The actual activity of SAP is approximately 500 U/cm^3 for all experiments.

3.3.4 FTIR-ATR Measurements

FTIR-ATR spectroscopy (MIDAC, M Series) was used to determine alterations in enzyme secondary structure induced by drying. The FTIR-ATR spectrums, based on the observation of the decrease in absorption bands which are formed by the passage of the ray through the sample, were taken as a result of the reflections between the crystal and the sample. The ATR accessory of the FTIR contained a ZnSe crystal and six reflectors with 45^0 angle.

CHAPTER 4

RESULTS AND DISCUSSION

This study was performed to obtain stable and storable SAP powders. In this context, the effect of air inlet temperature of the spray drier on SAP activity was evaluated. Thereafter, the effect of protective additives, glucose and maltodextrin, on SAP activity was tested during spray drying. Lastly, structural change of SAP during drying and storage stability of obtained SAP powders at 4^0C for a long period (6 months) were investigated.

4.1 Effect of Temperature

In order to evaluate the effects of temperature on SAP during drying, initial experiments were carried out in the absence of any protective agent. As seen in Figure 4.1, increasing air inlet temperature lead to an increase in activity loss of SAP during drying. The highest activity loss, 32% was observed at temperature 130^0C and the minimum activity loss was 8% at 70^0C.

In literature, Broadhead et *al.* (1994) investigated the effect of drying temperature on the residual activity of β-galactosidase and they observed activity losses even at the minimum drying temperature, 60^0C. Belghith et *al.* (2001) showed that high inlet-outlet temperatures had a negative effect on enzyme (cellulases) recovery. Vasiljevic and Jelen in 2003 investigated the

retention of β-galactosidase activity after spray drying at three different outlet temperatures and they stated that lower outlet air temperatures during spray drying resulted in higher β-galactosidase activity retention than at higher temperatures.

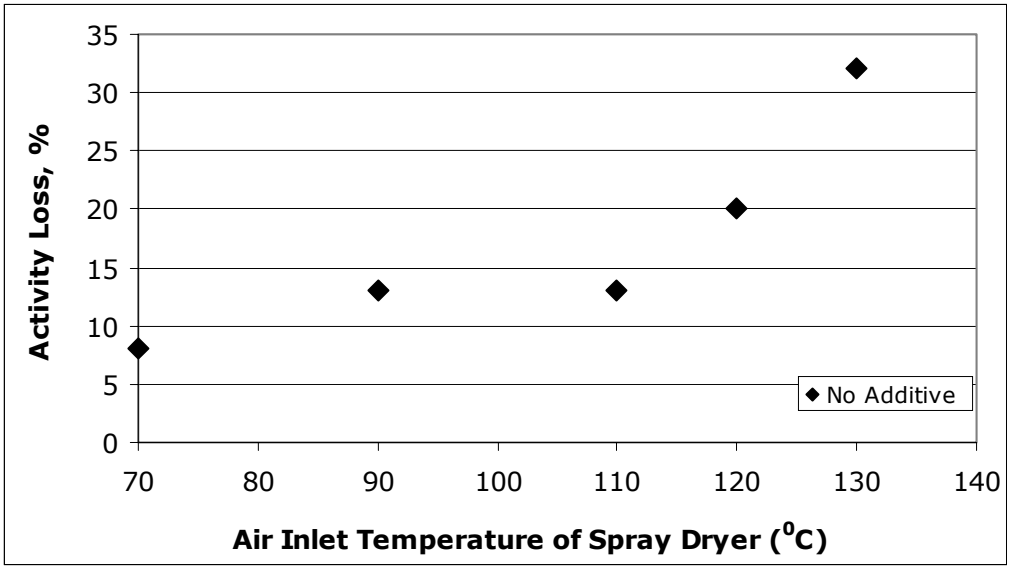

Figure 4.1 Effect of temperature on activity loss of SAP during drying

It has been reported that the enzyme activity is partially lost during spray drying may be due to thermal denaturation. However, considering the drying process in more detail, extensive thermal damage to the protein seems less likely. Spray droplets and the dried powder particles will maintain a temperature well below the inlet temperature of the drying gas throughout the process. As long as water evaporates from the droplets, a cooling effect will be achieved, which prevents the temperature of the drying material to rise above the wet bulb temperature of the drying air. Only in the last stages of drying, when the water activity of the drying material is low, can the temperature of the particles rise

above this temperature, but will still remain below the temperature of the air leaving the spray dryer (typically 15-20 ^{0}C lower) (Masters,1985).

In this study, dryer outlet temperatures were 56, 64, 76, 82, 90^{0}C due to the working inlet temperatures 70, 90, 110, 120, 130^{0}C. According to the literature, the denaturation temperature of SAP is around 70^{0}C(Çalık et *al*., 2001). It seems that only at higher temperatures SAP activity will be lost. However, even at the lowest operation temperature, 70^{0}C small activity loss was observed indicating adverse effects of the temperature on the enzyme structure start to play a role even at this relatively low temperature.

In addition to thermal denaturation, some other activity loss sources during drying are shear forces in the spray nozzle, extremes of pH and protein adsorption at the powder surface. To reduce the degree of activity loss of an enzyme during drying, stabilizing agents e.g. carbohydrates can be used. Two major mechanisms have been put forward that can (at least in part) explain the stabilizing effects of different carbohydrates: (i) carbohydrates replacing water with interactions with the protein and (ii) carbohydrates providing a glassy matrix. The 'water replacement' hypothesis states that in order to preserve the native structure of a protein, the hydrogen bonds formed between the protein and water molecules in an aqueous solution need to be replaced by new hydrogen bonds in the dry state. Carbohydrates have indeed been shown to form hydrogen bonds with proteins in the solid state. The 'glassy state' hypothesis states that it is essential to maintain the excipient in an amorphous state, which will be prevent the protein from changing its shape due to the rigidity of the matrix (Millqvist-Fureby et al., 1999a). Therefore, subsequent studies were performed by using glucose and maltodextrin as stabilizing additives and the effect of additives on activity loss of SAP during drying was evaluated.

4.2 Effect of Glucose

To preserve the native structure thus activity of SAP during drying, firstly glucose was used as a protective agent. Activity loss can be prevented by using glucose that remains in the amorphous phase with the protein and hydrogen bond to protein in the place of water during drying (Depaz et al., 2002). In this context, enzyme solutions with variable glucose concentrations were prepared and dried. Figure 4.2 summarizes the effect of glucose on activity of SAP during drying. In the presence of 0.5% glucose, only at 70^0C SAP activity was completely preserved. With the increase in glucose concentration up to 1%, activity loss of SAP decreased at the studied temperatures below 120^0C. At 2% glucose content, activity preservation was observed only at 90^0C.

The enzymes in aqueous solutions are generally stabilized by osmolytes, such sugars, some amino acids and salts due to preferential exclusion. However, the mechanism of the enzyme protection from denaturation and subsequent loss of activity during dehydration appeared to be rather complex. Depending on the kind of stress imposed, two theories have been proposed: water replacement and the glassy state. (Millqvist-Fureby at *al.*, 1999a,b; Depaz et al., 2002; Vasiljevic and Jelen, 2003)

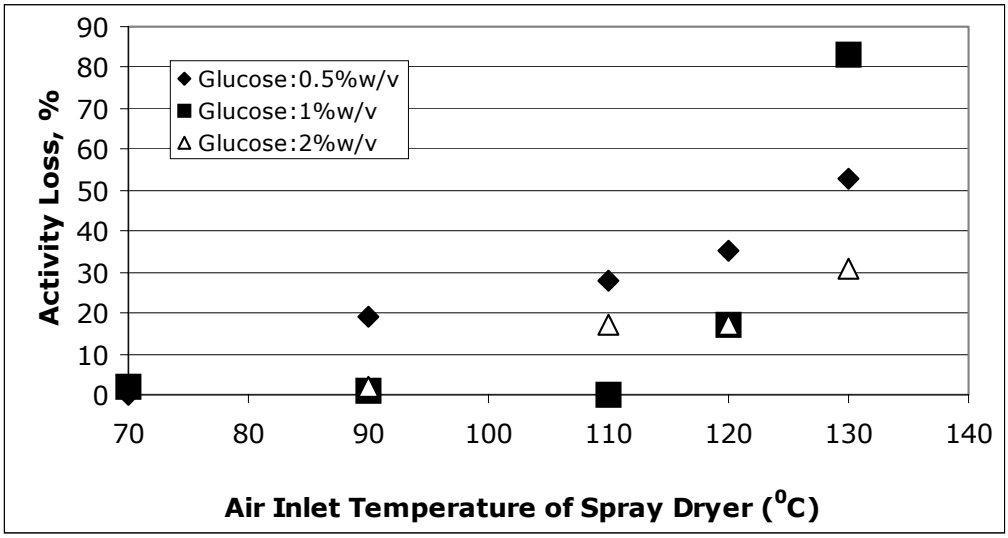

Figure 4.2 Effect of glucose on activity loss of SAP during drying with air inlet temperature

The nature of the sugars added had a profound effect on the enzyme activity preservation. Upon dehydration, the enzymes undergo conformational changes resulting in the loss of activity. Such changes may be prevented by sugar addition trough either water replacement or glassy state due to the physical entrapment of the enzyme into an amorphous sugar matrix. Millqvist-Fureby at *al.*(1999a) investigated the interactions between trypsin and various carbohydrates, i.e. lactose, sucrose, mannitol, cyclodextrin and dextrin during spray-drying. They showed that lactose readily attained the amorphous form, while sucrose acquired a varying degree of crystallinity which has a detrimental effect on enzyme activity. Depaz et al. (2002) examined the effect of saccharides on the stability of subtilisin during drying and they stated that the formulations containing sucrose and trehalose result in a more native-like secondary structure, presumably because they protect the enzyme during via hydrogen bonding. Vasiljevic and Jelen (2003) investigated the protective role of sugars (lactose, cellobiose and sucrose) in enzyme (β-galactosidase) activity preservation. They observed that all sugar-containing preparations resulted in

substantially higher β-galactosidase activity in the dry powders. Among them, lactose 5% (w/w) concentration was showed better enzyme activity preservation might have been due to enzyme substrate-binding, which consequently might have preserved the active center conformation of the enzyme, resulting in the high enzyme activity preservation. They speculated that at 5% sucrose concentration, the content of the amorphous fraction was not enough to prevent the enzyme deactivation resulting in substantially lower β-galactosidase activity in comparison to lactose at the same concentration. Increasing sucrose concentration to 10% would have resulted in higher content of the amorphous segment, possibly explaining the higher enzyme activity preservation, which was similar to that achieved by lactose at equal concentration.

Present study showed that activity preservation was totally achieved at moderate temperatures by increasing glucose concentration. It can be speculated that high activity preservation may be due to high amorphous content fraction and hydrogen bonding between enzyme and glucose. Interestingly, at high air inlet temperatures, increasing glucose concentration had a negative effect on SAP activity. According to the literature, it can be said that at high inlet temperatures when high concentrations of glucose was used, interaction between the sugar and protein was decreased (Carpanter and Crowe, 1989).

4.3 Effect of Maltodextrin

After the effect of glucose was investigated, another protective agent (maltodextrin) was used to evaluate the SAP activity during drying. The variations of activity loss of SAP with the air inlet temperature of the dryer in the presence of MD are given in Figure 4.3. At all concentrations of MD, high activity preservation was observed at 90^0C and 110^0C. Increasing air inlet temperature of the dryer up to 120^0C lead to an increase in activity loss. Interestingly, further

increase resulted with a decrease in activity loss of SAP at all concentrations of MD.

Since protein denaturation during drying requires a conformational change, it seemed reasonable to expect that protein (enzyme) stability should be greatly enhanced in a solid matrix, because formation of a glassy solid state results in significant arrest of transitional molecular motion and chemical reactions (Schebor et al., 1996). Note that at 130^0C drying hence going into the solid phase should occur at a faster rate. So this may outweigh the negative impact of the high temperatures especially at this particular temperature value.

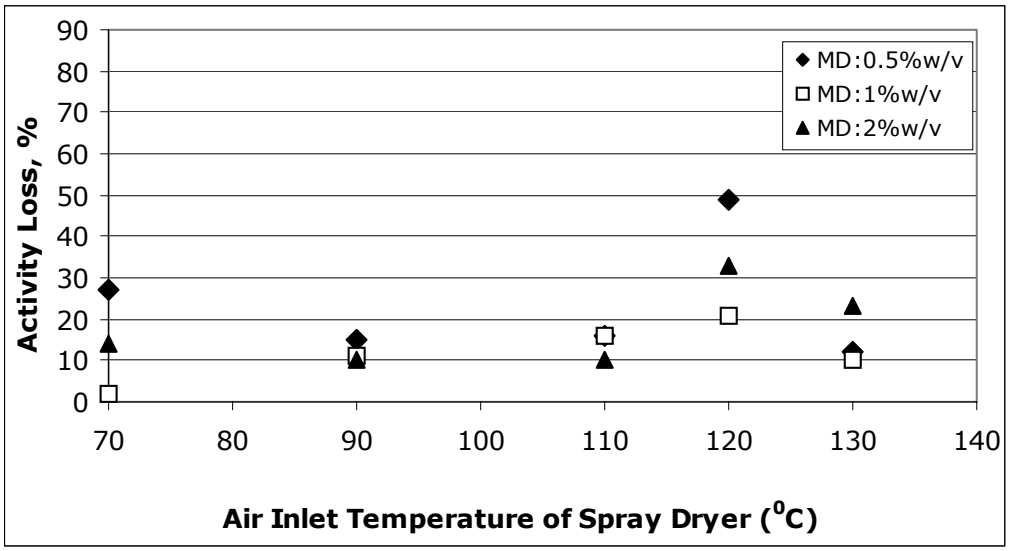

Figure 4.3 Effect of maltodextrin on activity loss of SAP during drying with air inlet temperature

4.4 Comparison of Protective Additives

The obtained data in Figure 4.4 was presented to determine the optimum condition for spray drying of SAP. As it can be seen in figure, at 70, 90 and 110^{0}C, the activity loss of SAP was greatly eliminated in the presence of glucose compared to MD. On the other hand, addition of MD was better than glucose for activity preservation of SAP at 130^{0}C, since glucose had a negative effect on SAP activity at that temperature. However, MD addition was not efficient so much to inhibit activity loss of SAP at moderate temperatures. On the basis of these results, 1% glucose addition is the best to preserve activity of SAP during spray drying within the studied range of concentrations of additives.

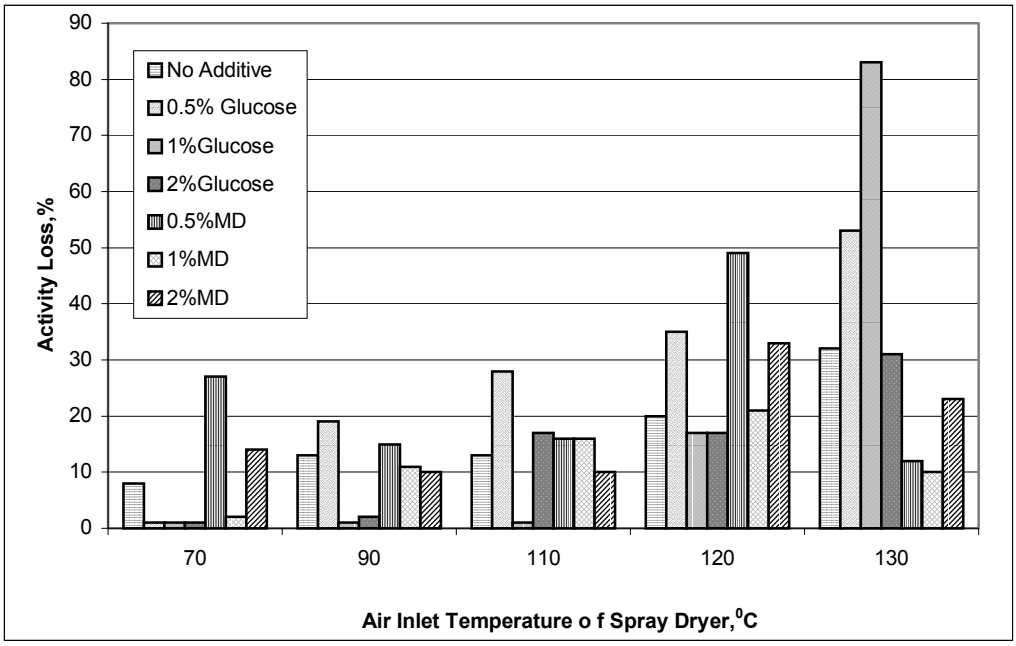

Figure 4.4 Comparison of additives for the activity preservation of SAP

Carpanter and Crowe in 1989 studied the interaction of stabilizing carbohydrates with dried proteins and they stated that when high concentrations of sugar was used, interaction between the sugar and protein was decreased and the greatest degree of protection was noted with intermediate amounts of sugar. Later on, Yamamoto and Sano (1991) resulted that the enzyme retention becomes lower with increasing molecular weight of sugars, which corresponds to the decrease of number of hydroxyl groups per molecule or the increase of hydrophocity. Since MD is a large molecule than sucrose, inactivation rates in MD is higher than sucrose. With the agree of this result, Tanaka et *al.* (1991) stated that when glucoside chains of saccharide are long, the protective activities on the basis of their weights decrease, since saccharides affect the ordering degree of water around proteins (solvent-mediated interaction).

According to the literature, it can be said that, preservation of SAP activity is well achieved with intermediate amounts of sugar, 1% glucose, due to the complete interaction between glucose and SAP via hydrogen bonding. Decrease in SAP activity in the presence of MD, which is a large molecule than glucose, can be explained due to the decrease in number of hydroxyl groups per molecule.

4.5 Structural Change of SAP in The Presence of 1% Glucose

The physical structure of the dried powder is very important, since the functionality of the powder largely depends upon it. To investigate the structural change of SAP during drying, the effects of drying on SAP secondary structure and the mechanism of the interaction of glucose and dried SAP powders was investigated in the presence of 1% glucose using FTIR. In Figure 4.5 some shifts in bands and change in the peak heights were observed, indicating possible structural change of SAP during drying with the increase in air inlet temperature.

As shown in figure, at elevated temperatures (120^0C (Blue line) and 130^0C (Red line)), spectral changes become more pronounced. The denatured SAP spectrum (yellow line) showed a pronounced decrease in the absorbance and major shifts in bands at 1399 cm^{-1}, 1237 cm^{-1} and 1061 cm^{-1}. So one may conclude that the impact of the drying at high inlet temperatures on the SAP structure becomes more severe than low inlet temperatures. When compared to the other spectrums and spectrum of pure SAP (dark blue line), at 90^0C (white line) more native-like structure for dried SAP powders was observed. As mentioned before, enzyme activity loss was almost prevented at 70, 90 and 110^0C air inlet temperatures in the presence of 1% glucose. When activity measurements were compared with FTIR results in the presence of 1% glucose, it can be said that high activities were observed when structural changes were rather limited.

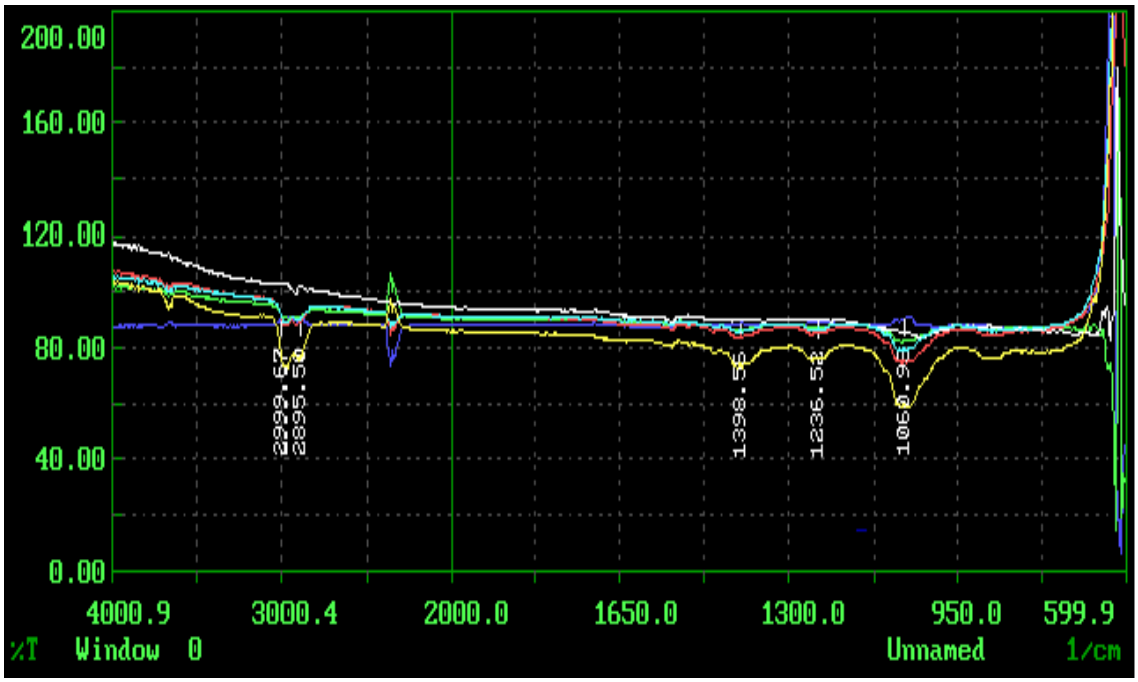

Figure 4.5 Infrared spectrums of spray dried SAP in the presence of 1% glucose. Dark blue line: pure SAP; Green line: 70^0C; White line: 90^0C; Blue line:120^0C; Red line:130^0C; Yellow line: denatured SAP.

4.6 Effect of Storage Time

The stability of obtained SAP powders during storage at 4^0C for up to six months was investigated. Table 4.6 demonstrates the effect of storage time on activity of SAP powders. In general, at high inlet temperatures, the activity of SAP powders was completely preserved in the presence of both glucose and MD. At low inlet temperatures, addition of 1% glucose enables the best stabilization. When considering all conditions, 0.5% MD addition was the best for stabilizing SAP powders.

Belghith et al. in 2001 investigated the effect of additives, MD and PEG, on the stability of enzyme cellulases during storage at 4^0C and 30^0C. As a result, 1%(w/v) MD completely stabilized cellulases even after a long period (8 months) but 1%(w/v) PEG had a negative effect on enzyme stability. Vasiljevic and Jelen (2003) investigated the activity retention of β-galactosidase powders, which was obtained after spray drying at three different outlet temperatures (40^0C, 50^0C and 60^0C), during storage at 7^0C after 30 days. They stated that the stability of the enzyme activity was significantly correlated to water activity of dry powders and processing at lower temperatures during spray drying resulted higher water activity and higher moisture content thus higher β-galactosidase activity loss.

When compared to the obtained results with literature, the activity stabilization at high inlet temperatures can be due to the low water activity and low moisture content. Effect of additives on SAP activity stabilization may be explained by amorphous matrix which forms between SAP and additives during drying.

Table 4.6 Effect of storage time on activity of SAP powders at 4^0C for up to 6 months

Condition	Activity Loss, %*
No Additive	30
0.5%Glucose; 70^0C	20
0.5%Glucose; 90^0C	11
0.5%Glucose; 110^0C	No Activity Loss
0.5%Glucose; 120^0C	No Activity Loss
0.5%Glucose; 130^0C	No Activity Loss
1%Glucose; 70^0C	14
1%Glucose; 90^0C	6
1%Glucose; 110^0C	No Activity Loss
1%Glucose; 120^0C	No Activity Loss
1%Glucose; 130^0C	No Activity Loss
2%Glucose; 70^0C	21
2%Glucose; 90^0C	14
2%Glucose; 110^0C	13
2%Glucose; 120^0C	9
2%Glucose; 130^0C	8
0.5%MD; 70^0C	No Activity Loss
0.5%MD; 90^0C	No Activity Loss
0.5%MD; 110^0C	No Activity Loss
0.5%MD; 120^0C	No Activity Loss
0.5%MD; 130^0C	No Activity Loss
1%MD; 70^0C	17
1%MD; 90^0C	8
1%MD; 110^0C	No Activity Loss
1%MD; 120^0C	No Activity Loss
1%MD; 130^0C	No Activity Loss
2%MD; 70^0C	18
2%MD; 90^0C	No Activity Loss
2%MD; 110^0C	No Activity Loss
2%MD; 120^0C	No Activity Loss
2%MD; 130^0C	No Activity Loss

$$* \text{ Activity Loss, \%} = \frac{\text{activity of SAP after storage}}{\text{activity of SAP before storage}}$$

CHAPTER 5

CONCLUSION

The results of the experiments allow one to draw the following conclusions.

1. Increasing air inlet temperature lead to an increase in activity loss of SAP during drying. The highest activity loss, 32% was observed at temperature 130^0C and the minimum activity loss was 8% at 70^0C.

2. In the presence of 0.5% glucose, only at 70^0C SAP activity was completely preserved. With the increase in glucose concentration up to 1%, activity loss of SAP decreased at the studied temperatures below 120^0C. At 2% glucose content, activity preservation was observed only at 90^0C. At high air inlet temperatures, increasing glucose concentration had a negative effect on SAP activity.

3. At all concentrations of maltodextrin, high activity preservation was observed at 90^0C and 110^0C. Increasing air inlet temperature of the dryer up to 120^0C lead to an increase in activity loss. Further increase resulted with a decrease in activity loss of SAP at all concentrations of MD.

4. In the comparison of two additives, the activity loss of SAP was greatly eliminated in the presence of glucose at 70, 90 and 110^0C. On the other hand, addition of MD was better than glucose for activity preservation of SAP at 130^0C,

since glucose had a negative effect on SAP activity at that temperature. However, MD addition was not efficient so much to inhibit activity loss of SAP at moderate temperatures. On the basis of these results, 1% glucose addition is the best to preserve activity of SAP during spray drying.

5. From FTIR-ATR spectrums, with the increase in inlet temperature of the drier some shifts in bands was observed, which shows the structural change of SAP during drying. At elevated temperatures (120^0C and 130^0C), damage to SAP structure during drying appears to be more severe and at 90^0C more native-like structure for dried SAP powders was observed when compared to the spectrum of pure SAP and other spectrums. When activity measurements were compared with FTIR results in the presence of 1% glucose, it can be said that high activities were observed when structural changes were rather limited.

6. In general, at high inlet temperatures, the activity of SAP powders was completely preserved in the presence of both glucose and MD during storage at 4^0C for up to six months. At low inlet temperatures, addition of 1% glucose enables the best stabilization. When considering all conditions, 0.5% MD addition was the best for stabilizing SAP powders.

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APPENDIX A

CALIBRATION OF CELL CONCENTRATION

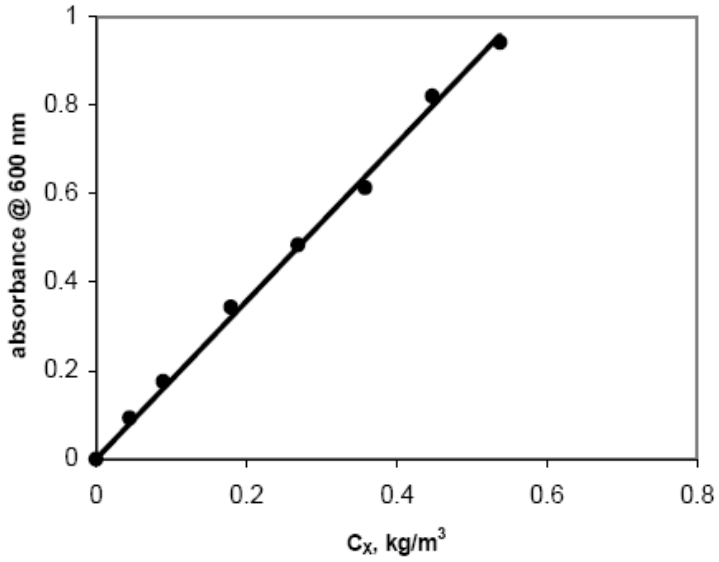

Figure A.1 Calibration curve of cell concentration

Slope of the calibration curve, m=1.782 1/kg m^{-3} (λ=600 nm)

$$Cx = \frac{Absorbance}{1.782} \times DilutionRate$$

APPENDIX B

CALIBRATION OF SERINE ALKALINE PROTEASE ACTIVITY

The slope of the calibration curve is m= 0.8 1/(μmole cm^{-3}). 1U is defined as 4nmole tyrosine Per minute and the enzymatic reaction takes place for 20min. The activity;

$$A = \frac{A_\lambda}{m} x DilutionRate \frac{1U}{4nmole\,\min^{-1}} x \frac{1}{20}$$