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Effect of Lyophilization Conditions of Recombinant L-phenylalanine Ammonia-lyase on Enzyme Properties

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Abstract: The effect of moisture content and status of the recombinant L-phenylalanine ammonia-lyase preparation on the thermo-physical characteristics of the enzyme (thermal diffusivity, thermal conductivity, density, mass heat capacity) is defined. The effect of the freezing process of L-phenylalanine ammonia-lyase on its properties is studied. It is shown that after a single freezing-unfreezing cycle the loss of enzyme activity is 12,0%. The optimal activity stabilizer for L-phenylalanine ammonia-lyase lyophilization-0,5% solution of D-trehalose-is chosen. The cryoscopic temperature depending on the enzyme protein concentration is evaluated. It is shown that the crystallization of the enzyme preparation in general corresponds to the character of aqueous sodium chloride solution crystallization. The optimal temperature for the L-phenylalanine ammonia-lyase drying process conducting (20-40°C) was obtained. It is proved that lyophilization of the enzyme at the selected temperature has no effect on the microstructure of the preparation.

Key words: L-phenylalanine ammonia-lyase · Lyophilization · Freeze-drying · Enzyme activity · Thermo-physical properties · Moisture · Cryoscopic trremperature

INTRODUCTION

L-phenylalanine ammonia-lyase (PAL, CF 4.3.1.5) catalyzes the reversible reaction of amino acid deamination of L-phenylalanine to trans-cinnamic acid and ammonia [1]. The enzyme is of interest as a therapeutic agent for the treatment of phenylketonuria. It can be used for both the direct treatment of phenylketonuria and phenylalanine-free foodstuff production [2]. Apart from the medical application, the enzyme can be used in biotechnology for the production of L-phenylalanine from trans-cinnamic acid [3].

The main source of this enzyme is yeast cells [4]. It seems to be promising to use the recombinant strain Escherichia coli for industrial production of enzyme [5].

One of the important steps in recombinant enzyme production is dehydration. Production of biopharmaceutical protein preparations is complicated by their limited physical and / or chemical stability. Chemical and biological stability is one of the most important characteristics for enzymes. Effective way to improve the stability of enzymes is drying by lyophilization. Lyophilization can reduce the moisture content in the material up to 3%. Currently most commercial pharmaceutical products on the market are dehydrated by lyophilization. However, freeze-drying can have a negative impact on the protein fraction of the preparation. Unfortunately, even successfully performed lyophilization does not guarantee the required quality characteristics and stability of enzymes [6].

Properties of the finished product are mostly dependent on operating parameters of lyophilization process. Enhancing energy efficiency of freeze-drying is not always accompanied by a corresponding increase in quality indicators of lyophilized products. Therefore, lyophilization processes of enzymes need optimization and rationalization of operational parameters. Rational mode of freeze-drying must ensure a minimum duration and power consumption of the process under the best indicators of quality of dried material. Duration of lyophilization depends primarily on the intensity of drying, i.e. the amount of moisture removed per unit of surface area of dryable material per unit of time. Despite the long history of dry biopreparation production, a simple, low-cost and reasonably accurate methods of calculating operating parameters of freeze-drying and its separate stages has not been developed yet. It happened because of the need of physical and mathematical

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modeling of freeze-drying processes for complex bio-organic systems. Mathematical apparatus used for the construction of such models is too bulky, the boundary conditions are ambiguous and, in addition, there is no unified approach to the construction of mathematical models of processes for freeze-drying of different biomaterials [7].

The objective of this paper is to investigate of L-phenylalanine ammonia-lyase lyophilization conditions on the enzyme properties.

MATERIALS AND METHODS

In this paper L-phenylalanine ammonia-lyase obtained by genetic engineering using the pal gene, isolated from Rhodosporidium toruloides is used [8].

Determination of Protein Concentration: Protein concentration was determined spectrophotometrically by absorbance at 260nm and 280nm wave range using Warburg-Christian method.

Determination of L-phenylalanine Ammonia-lyase Activity: The method published by Sigma was used to determine PAL activity.

Determination of Resistance to Enzyme Freezing: An aliquot of the purified protein was diluted in 0.1 M Tris-HCl buffer, pH 8,5. Part of aliquot was used for determination of enzyme activity and protein concentration. The remaining portion of 1,5 ml was frozen in air medium in natural convection at-18°C and kept at this temperature for 12 hours. After that the frozen formulation was thawed at 4°C and activity was determined.

The Effect of Low Temperatures on the Quality Parameters of the Protein Preparation of Repeated Purification. Sulphate suspension was centrifuged and the resulting precipitate was dissolved in 50 mM Tris-HCl buffer, pH 8,5. It was then dialyzed against the same buffer (with two changes of buffer) for 20 hours. Equal amounts of protein (225 µl each) was placed in three glass vials of 0,5 ml. 25 µl of 5% aqueous solution of D-Trehalose was added to the one vial, 25 µl of 5% aqueous solution of polyvinylpyrrolidone to another and 25 µl of 50 mM Tris-HCl, pH 8.5 to the third one. Preparation activity after dialysis was used as control. Vials were closed with several layers of gauze, frozen in air medium at natural convection and-70°C and kept at this temperature for 12 hours. The vials were placed in a pre-cooled freeze dryer "Iney-6" (Russia). The drying was performed for 6 hours at 6 Pa vacuum,-40°-40°condenser temperature and 20°C shelf temperature. Dried preparations in vials was dissolved in the original volume of deionized water (250 µl) and the specific activity of the protein was determined.

RESULTS AND DISCUSSION

Lyophilization is a complex technological process, on which the quality product parameters depend. Rational regimen of freeze-drying allows to keep physical and chemical characteristics of the dried object at the minimal energy costs.

Optimal regime determination of product freeze-drying, including enzyme preparations, should begin with the physico-chemical and thermal properties study. The initial data was information about the chemical composition of the L-phenylalanine ammonia-lyase tested preparation (Table 1).

Study of the enzyme preparation thermal characteristics was performed by the first buffer method of the two temperature-time intervals [9]. The thermal preparation characteristics was determined in the liquid, frozen and dehydrated state. Studies The results of the thermophysical L-phenylalanine ammonia-lyase characteristics are shown in Table. 2.

The presented data show that the thermal phenylalanine ammonia-lyase preparation characteristics is largely determined by the moisture content. The thermal conductivity of the preparation during freezing increases 4 times more, in proportion to an increase of ice thermal conductivity by freezing water. Under the dehydration the dry preparation thermal conductivity increases 6 times more. This is due to the high thermal conductivity of sodium chloride, which is 6.5 W K) and its high content in?/ (m the dehydrated preparation (45,5%).

Heat capacity reduces at freezing in 2 times. When dehydrating it reduces slightly, because the value of the heat capacity preparation dry residue component commensurate with the ice heat capacity.

Table 1: Physicochemical properties of the enzyme preparation of L-phenylalanine ammonium-lyase

Protein content, mg / ml	10,0
Yeast extract content,%	0,5
NaCl content, %	1,0
Specific activity, unit/mg of protein	3,0

Table 2: Thermo-physical properties of L-phenylalanine ammonium-lyase before and after freezing

Condition	Thermal diffusivity $a \cdot 10^7$, m^2/s (±5%)	Thermal conductivity λ, W/(m•K) (±5%)	Density ρ, kg/m^3 (±2%)	Mass heat capacity C_m, J/(kg•K) (±5%)
Liquid preparation (t=18°C)	1,37	0,56	1013	4166
Frozen preparation (t=-24°C)	11,97	2,18	920	1979
Dry preparation (t=20°C)	14,94	3,36	1192	1888

Table 3: Effect of freezing on L-phenylalanine activity

Condition	Specific activity, E/мг	Activity loss, %
Before freezing	3,00	0,0
After unfreezing	2,64	12,0

Table 4: Effect of solvents on PAL activity during the freezing process

Sample	Specific activity, E/mg of protein	Activity loss, %
Before drying (control)	2,99	0,0
Only buffer	2,58	13,8
Trehalose 0,5%	3,00	0,0
Polyvinylpyrrolidone 0,5%	2,83	5,3

Table 5: Effect of the PAL enzyme preparation soluble components concentration on the cryoscopic temperature

Mass fraction of solids, %	Mass fraction of protein, %	NaCl, %	Cryoscopic temperature, °C
2,2	1,14	1,00	-0,55
4,8	2,49	2,18	-1,26
8,4	4,35	3,82	-2,32
13,3	6,89	6,05	-3,98
18,9	9,79	8,59	-6,16

The temperature conductivity increases by 9 times when freezing. When dehydrating-by 20%.

The freezing process is a part of the freeze-drying technology. It is known that enzyme agents are thermolabile products. Therefore the research of the freezing process and its effect on the L-phenylalanine ammonia-lyase properties is of interest.

Since the preparation PAL is more stable in the alkaline pH area, the freezing and lyophilization experiments were conducted at pH 8,5.

Firstly we tested the enzyme stability to freezing. It is found that when the single cycle freezing-defrosting, the loss of enzyme activity were 12,0% (Table 3).

Then we examined the effect of low temperatures on the quality parameters of protein preparation double purification. It is shown that the loss of enzyme activity during dialysis did not occur. The dialyzed preparation had a specific activity of 2,99 U / mg protein, the protein concentration was 8,51 mg / ml. The assessment results of the solvents impact on the PAL activity during freezing are shown in Table. 4.

Thus, the best stabilizer of the PAL activity on lyophilization is 0,5% solution of D-trehalose. Polivililpirrolidon has little effect at the same concentration. Besides, its removal from protein preparations is a time consuming procedure.

An important indicator of the dried in the process of freeze-drying is the freezing point depression temperature-the temperature of the crystallization beginning contained in the water object. The temperature of the freezing point depression is a necessary parameter in the study and design of low-temperature biomaterials treatment processes. The freezing point depression temperature of the aqueous solutions depends on the type and quantity of soluble components.

The protein concentration affects the point depression temperature at the freezing. Furthermore, since the enzyme has a significant proportion of the yeast extract and sodium chloride, investigated temperature the dependence of the point depression temperature of enzyme preparation on the concentration of these components (Table 5).

The results were compared with the freezing point depression temperature of the chloride solution aqueous sodium and it was found that the enzyme preparation nature of the crystallization corresponds to the character of the chloride solution aqueous sodium crystallization. However cryoscopic temperature the enzyme preparation is slightly higher (by 0,1-0,2°C) than the corresponding by the aqueous solution concentration of the sodium chloride. This is due to the hydrophobic properties of the protein enzyme preparation. However, the effect is little, so that as a basis for modeling basis when enzyme preparation L-phenylalanine ammonia-lyase freezing an aqueous solution of sodium chloride may be used.

The results of the enzyme cryoscopic temperature dependence on the protein concentration in the preparation is shown in the equation:

$$t_{kp} = 1{,}0381 \cdot \ln(x) - 3{,}8539 \quad (1)$$

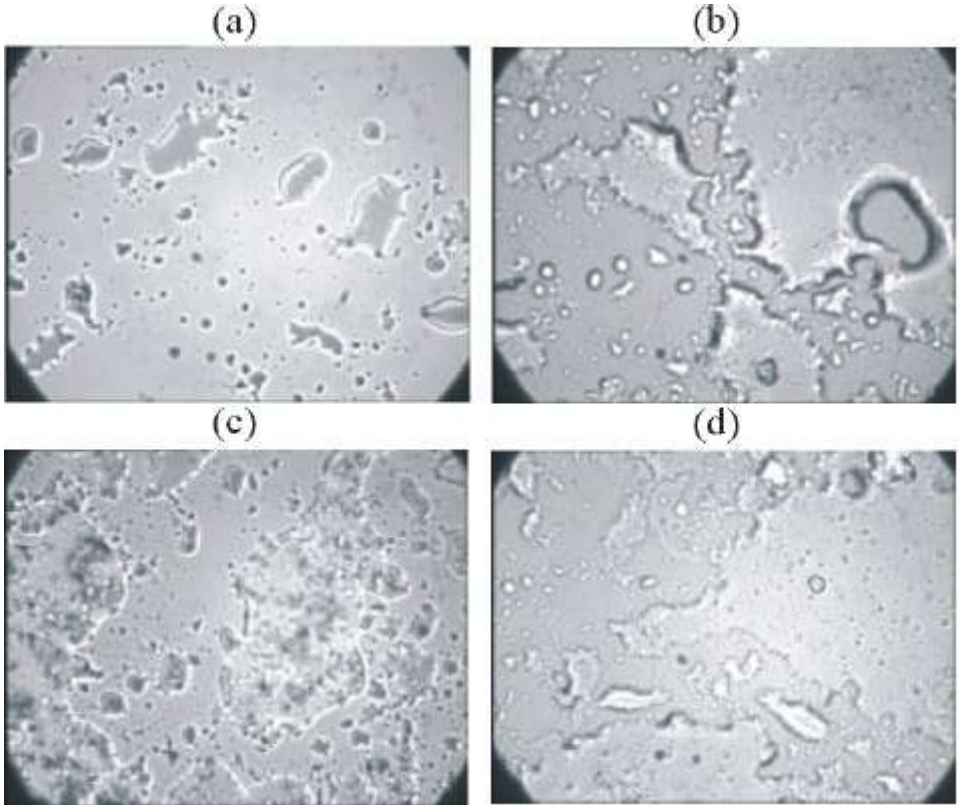

Fig. 1: The microstructure of the dried enzyme preparation (500-fold increase) at different temperatures of stabilization of the dried layer surface: a-15°C; b-20°C; c-30°C; d-40°C

Table 6: Amount of moisture removed from the enzyme preparation during freeze-drying

	Moisture (%) of the total amount of moisture removed during drying, °C				
Freeze-drying period	5	15	20	30	40
Vacuuming systems	19	22	20	19	19
Sublimation	42	53	55	57	62
Warming-up	38	25	23	20	17
Total time, min	410	375	330	270	240

It shows that the protein concentration change in the formulation from 3.5 to 7,5 mg / ml, leads to the freezing point depression temperature change by 0,3-0,6 ° C, when the protein concentration is from 7,5 to 11,5 mg / ml the freezing point depression temperature changes by 0,6-1,3°C.

We studied the moisture quintaty removed form the PAL enzyme preparation during freeze-drying (Table 6).

Table. 6 shows that the increase of dried layer temperature stabilization level causes the increase of the moisture quantity, removed during sublimation and the decrease of the moisture quantity, removed during the warm-up. The temperature of 20-40°C is a most favorable for the enzyme preparation L-phenylalanine ammonia-lyase drying process, since at this temperature level the moisture removal of the enzyme preparation is enhanced and the drying time is reduced.

When freeze drying the lyophilized preparation microstructure is of interest. Fig. 1 shows the microstructure of the L-phenylalanine ammonia-lyase preparation, obtained by freeze-drying method at different temperatures to stabilize the surface of the dried layer.

Fig. 1, shows that a dried enzyme L-phenylalanine ammonia-lyase preparation, produced by the sublimation method, contains the particles approximately of the same size with hard and smooth surface.

CONCLUSION

The effect of lyophilization conditions of recombinant L-phenylalanine ammonia-lyase on enzyme properties was studied. Thermal and physical characteristics of the enzyme preparation of L-phenylalanine ammonia-lyase in liquid, frozen and dry conditions were determined. The effect of freezing

temperature on the activity of the enzyme preparation of L-phenylalanine ammonia-lyase was investigated. Dependence of the cryoscopic temperature of the enzyme preparation on the concentration of its soluble components was assesed. The amount of water removed from the enzyme preparation during freeze-drying was investigated. The microstructure of the enzyme preparation before and after freeze-drying was examined with an electron microscopy.

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