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Obtaining higher transesterification rates with subtilisin Carlsberg in nonaqueous media

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Abstract—Three phase partitioning (protein precipitate obtained as an interfacial layer between lower aqueous and upper t-butanol phases, formed by the addition of ammonium sulphate and *t*-butanol to the aqueous solution of protein) followed by lyophilization in the presence of two-component excipient resulted in 400–480× increases in transesterification activity of lyophilized powders of subtilisin Carlsberg, depending on the solvent. The three phase partitioned enzyme, 'dried' by washing with butanol, gave 3–4× higher rates (depending on the solvent used) than the enzyme preparation dried by lyophilization in the presence of two-component excipient system.

The use of serine proteases like subtilisin Carlsberg in nonaqueous media for the synthesis of esters and peptides is well established.[1–3] Recent work has clearly shown that poor activity of enzyme powders in organic media is due to structural changes in enzyme molecules at the lyophilization stage.[4] Co-lyophilization with additives has been extensively attempted to obtain higher activity.[4–7] It is only recently that it has become clear that a protein is subjected to two different kinds of stress during lyophilization: freezing and drying.[8] It has been shown that a two-component excipient system provides the best protection to a protein during lyophilization. In this approach, one excipient [polyethylene glycol (PEG)] provides cryoprotection and the second excipient (a carbohydrate) acts as a lyoprotectant. In the present work, it is shown that the use of such a two-component excipient system during lyophilization led to subtilisin Carlsberg which showed higher transesterification rate, as compared to the unprotected enzyme or the enzyme colyophilized with just the lyoprotectant or cryprotectant.

Table 1 shows the effect of the presence of trehalose as lyoprotectant or the two-component system of cryoprotectant (PEG) + lyoprotectant (trehalose) during lyophilization on the activity of subtilisin Carlsberg in organic media.[9] n-Octane and t-amyl alcohol were chosen as the representative non-polar and polar media for evaluating the transesterification activity of protease.[10] The presence of the lyoprotectant enhanced the transesterification activity by 70 times in *n*-octane and by 61 times in t-amyl alcohol. The use of two-component excipient system, on the other hand, yielded lyophilized preparations, which enhanced the transesterification activity by 118 and 99 times, respectively, as compared to the activity of the enzyme lyophilized alone.

It has been shown that Proteinase K, when subjected to three phase partitioning, led to an enzyme preparation which showed 2.1 times higher caseinolytic activity in aqueous medium.[11] Three phase partitioning consists of adding ammonium sulphate and *t*-butanol to an aqueous solution of the enzyme resulting in its precipitation as an interfacial layer between aqueous and organic phases. It was found that subtilisin Carlsberg, after being subjected to three phase partitioning[12] and lyophilization, showed about four times higher caseinolytic activity in aqueous buffer[13] as well as transesterification activity in both *n*-octane and *t*-amyl alcohol.[12] The presence of the lyoprotectant and the two-component excipient system again offered protection to the enzyme which had been subjected to three phase partitioning (Table 2). For this enzyme preparation as well, the two-component excipient system offered higher protection and gave higher transesterification activity in both

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n-octane and *t*-amyl alcohol. Hence, effectively, this preparation showed 483 times higher transesterification activity over native subtilisin Carlsberg, which was lyophilized without any cryoprotectant/lyoprotectant. The corresponding enhancement in transesterification activity in t-amyl alcohol was 399 times.

However, it was found that even the two-component excipient system apparently did not prevent the loss in the activity during lyophilization of subtilisin Carlsberg subjected to three phase partitioning. The interfacial precipitate of the enzyme after three phase partitioning was washed with dry *t*-butanol to remove any water. This preparation (without any lyophilization) in *n*-octane gave a rate of 62.4 mmol h^{-1} mg^{-1} (1783 times than that given by subtilisin Carlsberg lyophilized without any additive). The corresponding rate in *t*-amyl alcohol was found to be 40.5 mmol h^{-1} mg^{-1} (1191 times the transesterification rate of the subtilisin Carlsberg lyophilized alone).

Drying by lyophilization consists of two stages—primary and secondary. It is the latter which is largely responsible for causing structural changes.[14] With growing awareness of this fact, numerous efforts have been made to obtain more active preparations for use in organic media. PEG is a known cryoprotectant and works by preferential exclusion from the protein surface.[8] However, PEG does not provide any protection during the drying stage of lyophilization. Drying is known to convert the native structure of proteins with predominantly β-sheet structures. This is attributed to the loss of water molecules which were originally hydrogen bonded. Upon the loss of these water molecules, peptide bonds get hydrogen-bonded to each other. In order to prevent this, the presence of a disaccharide like trehalose as a lyoprotectant is required. Such molecules form hydrogen bonds with the protein molecule and minimize structural changes due to dehydration stress. This has been the basis for the design of a two-component excipient system for protein lyophilization.[15] The results with subtilisin Carlsberg reported here show that the use of a two-component excipient system indeed gave a lyophilized preparation with higher activity than the lyophilized preparation obtained without any excipient or with lyoprotectant alone.

Table 1. Effect of additives during lyophilization on the rate of transesterification reaction catalyzed by subtilisin Carlsberg

	Initial reaction rate (mmol h^{-1} mg^{-1})	Fold increase
Reaction in *n*-octane		
Enzyme lyophilized alone	0.035	1
Enzyme colyophilized with 2% trehalose	2.49	70
Enzyme colyophilized with 2% trehalose + 1% PEG	4.18	117.6
Reaction in *t*-amyl alcohol		
Enzyme lyophilized alone	0.034	1
Enzyme colyophilized with 2% trehalose	2.08	61
Enzyme colyophilized with 2% trehalose + 1% PEG	3.38	98.8

Subtilisin (100 mg) was dissolved in 10 mL of 20 mM Tris HCl, pH 7.8 and the solution was colyophilized with different additives.

Table 2. Effect of additives during lyophilization on the rate of transesterification reaction catalyzed by three-phase partitioned subtilisin Carlsberg

	Initial reaction rate (mmol h^{-1} mg^{-1})	Fold increase
Reaction in *n*-octane		
Enzyme lyophilized alone	0.146	1
Enzyme colyophilized with 2% trehalose	9.93	68
Enzyme colyophilized with 2% trehalose + 1% PEG	16.89	116
Reaction in *t*-amyl alcohol		
Enzyme lyophilized alone	0.138	1
Enzyme colyophilized with 2% trehalose	8.28	61
Enzyme colyophilized with 2% trehalose + 1% PEG	13.58	98

The results also show that subtilisin Carlsberg, subjected to three phase partitioning, gave higher activity in organic medium containing low water as well. X-ray diffraction studies with Proteinase K, subjected to three phase partitioning, reported earlier had shown that three phase partitioning resulted in higher overall flexibility of the enzyme conformation.[11] This was the structural basis for the higher activity of the enzyme molecule in aqueous buffer. Presumably, the higher activity of subtilisin Carlsberg originates in similar structural changes. It seems that the structural changes are advantageous in terms of higher transesterification activity of the enzyme in organic media.

In these studies, two solvents of differing polarity were chosen. In agreement with the known behaviour of enzymes, higher activity was observed in the more nonpolar medium.[16] It is worth noting that subtilisin Carlsberg, subjected to three phase partitioning, showed the same trend.

The results also show that even the presence of the two-component excipient system failed to prevent inactivation completely. This is indicated by the fact that the TPP-treated enzyme, 'dried' by washing with butanol, gave much higher activity (3.7 times in *n*-octane and 3 times in t-amyl alcohol) in organic solvents, as compared to the preparation dried by lyophilization in the presence of the two-component excipient system. In fact, precipitation and washing by water-miscible organic solvents to obtain 'dry' enzymes for use in organic media is, by now, a well known strategy.[2,17]

Lyophilization has been the most frequently used method for drying enzymes and hence will probably continue to be used for this purpose. The results reported here show that the combined use of a lyoprotectant and cryoprotectant may, at least substantially, prevent structural damage to the enzyme for obtaining enhanced activity in organic media. Subjecting the enzyme to three phase partitioning before lyophilization

in the presence of a two-component excipient system may give still higher activity. Overall (as results with subtilisin Carlsberg show), this combined strategy is capable of giving an increase of about 1190–1783 times in the biological activity of enzyme in organic media. The use of KCl during lyophilization is reported to give much higher increase in activity[4] but somewhat different experience has been reported by other groups.[3,18]

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