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Improving Activity of Salt-Lyophilized Enzymes in Organic Media

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Abstract Lyophilization with salts has been identified as an important method of activating enzymes in organic media. Using salt-activated enzymes to transform molecules tethered to solid surfaces in organic phase requires solubilization of enzymes in the solvents. Methods of improving performance of salt-lyophilized enzymes, further, via chemical modification, and use of surfactants and surfactants to create fine emulsions prior to lyophilization are investigated. The reaction system used is transesterification of *N*-acetyl phenylalanine ethyl ester with methanol or propanol. Initial rate of formation of amino acid esters by subtilisin Carlsberg (SC) was studied and found to increase two to sevenfold by either chemical modification or addition of surfactants in certain solvents, relative to the salt (only)-lyophilized enzyme. The method to prepare highly dispersed enzymes in a salt-surfactant milieu also improved activity by two to threefold. To test the effect of chemical modification on derivatization of drug molecules, acylation of bergenin was investigated using chemically modified SC.

Keywords Subtilisin Carlsberg · Salt lyophilization · Nonaqueous media · Drug derivatization · Bergenin · Acylation · Surfactant · PEG

Introduction

Derivatization of drug candidates to prepare diversified libraries has traditionally been done via combinatorial chemistry. There have been several reports on using enzymes for drug derivatization, and this combined with high-throughput screens has led to the development of the field of combinatorial biocatalysis [1–3]. Derivatization of molecules with certain ligands such as acyl and aromatic moieties introduces the need to conduct reactions in

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organic media, which improves the solubility of the reacting substrates. However, enzymes typically have lower activities in organic media [4]. The increasing application of biocatalytic processes in pharmaceutical, food, and fine chemical industries has resulted in methods to improve the activity and stability of enzyme biocatalysts in organic media [5–7]. These methods include immobilization techniques [8]; stabilization via lyophilization with lyoprotectants [9], salts [10], or addition of surfactants [11, 12]; chemical modification with amphiphilic or hydrophobic polymers [13, 14]; and activation by addition of salts during lyophilization [10, 13].

The salt-based lyophilization method has shown activation of up to four orders of magnitude for enzymatic reactions in organic media [10, 13, 15]. Whereas this method improves activity, it does not improve solubility of the enzymes. Additionally, if the substrates are attached to surfaces, such as in microwell plates [16] to perform sequential derivatization, solubilization of enzymes would certainly help in increasing the rate of conversion of the attached substrates. The goal of this work was to investigate the effect of chemical modification and surfactant addition on transesterification rate of salt-lyophilized subtilisin Carlsberg (SC) in organic solvents. SC is a protease from *Bacillus licheniformis* capable of transesterifying alkyl esters. The chemical modification and surfactant addition can potentially assist in protein solubilization and thus improve the rate of the reaction. The enzyme was modified with polyethylene glycol (PEG) and an alkyl group, 14 carbons long, followed by salt-lyophilization to study the effect of chemical modification. Two surfactants, Aerosol OT (AOT) and Tween 20 (T 20), were studied to determine the effect on activity in various organic solvents. Bergenin was used as a model drug substrate to demonstrate enzymatic transformation in organic media.

Materials and Methods

Chemical Modification of Enzyme

The enzyme SC was modified with methoxy PEG (molecular weight 5,000) activated with p-nitrophenyl carbonate (Sigma, M-3903). The enzyme (240 mg) was dissolved in 100 mM potassium phosphate buffer pH 7.8 and stirred with 480 mg (~10 × molar equivalent) PEG for a period of 2 h. The reaction was monitored by release of the p-nitrophenyl group (absorbance at 405 nm). The modified enzyme was purified from the solution by washing with phosphate buffer in an ultrafiltration cell (Amicon, Model 8050) using a 30,000-Da molecular weight cut-off membrane, over several hours. The modified enzyme was then lyophilized as described below.

A double modification of SC with two groups, a C14 alkyl group and PEG, was conducted to obtain a PEG-C14-SC. The enzyme SC has 10 lysine groups accessible for chemical ligation. The PEG-C14-SC was prepared by first modifying the SC with methoxy PEG using a molar ratio of 1:2, enzyme/methoxy PEG, followed by modification with tetradecaldehyde. The first modification (with methoxy PEG) was conducted over a 2-h period, followed by washing in an ultrafiltration cell as described above. The procedure for the C14 modification consisted of dissolving 240 mg of SC modified with PEG in a 50:50 mixture of potassium phosphate buffer and ethanol, followed by addition of 400 mg of sodium cyanoborohydride, followed by the addition of 40.8 mg of tetraldehyde suspended in 2 mL ethanol. The mixture was stirred over a period of 2 h, followed by a washing step in an ultrafiltration cell to remove excess reagent.

Salt-Lyophilization of Enzyme

Native and modified SC were lyophilized with 0, 50, 75, and/or 99% salt concentration (in the lyophilized preparation). The salt mixture used contained 75% sodium bicarbonate and 25% sodium acetate by weight [13], with the total salt concentration at 1 M in the salt–SC solution used for lyophilization. The solution also contained 1 mg/mL potassium phosphate, pH 7.8, to buffer the lyophilized enzyme. The salt–SC solutions were lyophilized for a period of 36 h at −50°C and 8 μm Hg.

Enzyme Assays

The reaction system used for studying the activity of SC was transesterification of *N*-acetyl phenylalanine ethyl ester (APEE) with n-propanol or methanol. A solution of APEE was prepared in propanol and added into reaction vials giving a final propanol concentration of 0.85 mM and APEE concentration of 20 mM. The solvents studied were hexane, toluene, and methanol. In case of methanol, the reaction of interest was methanolysis of the ester. The total solvent volume used was 3 or 5 mL. Hexane and toluene were used after overnight saturation with distilled water, whereas methanol solvent used was prepared by adding 1 vol% deionized water to neat methanol. The reactions were carried out for a period of 4 h, to obtain a linear rate representing V_{max}. Aliquots of 300 μL were collected at five different time points between 20 min after the start of the reaction and the end of the run, and they were analyzed by gas chromatography (GC) or high-pressure liquid chromatography (HPLC). The concentration vs. time data between 20 and 240 min were plotted to obtain an initial rate of reaction. This plot was linear with R^2 between 0.90 and 0.99.

The effect of the surfactants AOT (Fischer Scientific) and T 20 (Sigma, P-7949) was assessed by adding these surfactants into the reaction vials at the beginning of the experiment as a solution or suspension in the solvent being studied. The amount of surfactant was equal to the amount of the enzyme–salt mixture (about 5–10 mg per reaction). The reactions were carried out in Teflon-lined screw-cap vials with shaking at 200 rpm and at 37°C with pyrene as an internal standard at 0.5 mM. A control with no enzyme was also run with each experiment. No conversion was obtained in the control reactions. The error in the rate calculations was estimated by obtaining a cumulative error in measurements that went into the rate equation. This included the experimentally determined slope of concentration vs. time plot (R^2 between 0.90 and 0.99), error in weighing the enzyme catalyst and assessment of enzyme concentration in the salt-enzyme preparation. The errors in these analyses were 1–10%, 0–4%, and 1–9%, respectively. This results in a total error in the range 2–23%.

Derivatization of Bergenin

Bergenin is a model compound for flavonoids [17] exhibiting several pharmacologically relevant effects including antioxidant, free radical scavenging, metal ion chelation, and nuclear type II estrogen-binding antagonism. Bergenin (Sigma, B-6776) was derivatized with ethyl acetate to obtain bergenin 4,11-diacetate and bergenin 4-acetate [2, 18]. The experiment was conducted by adding 200 mM bergenin dissolved in methanol into 20-mL scintillation vials. The solvent (methanol) was volatilized, followed by addition of 2 mL toluene containing 5% dimethyl sulfoxide (giving 2 mM bergenin in the reaction vessel).

The ethyl acetate concentration in the reaction vessel was 100 mM. The reaction was carried out by shaking at 200 rpm at 40°C for a period of 66 h.

Surfactant-Assisted Salt Enzyme Nanodispersions

A preparation of the SC was made by adding the surfactant to the enzyme prior to lyophilization. This was done as follows: The enzyme SC was dissolved in the buffered salt solution, which was then dispersed into a solvent by addition of surfactants. The solvent used in this experiment was hexanol and the surfactants studied were AOT and T 20. The amount of surfactant was equal to the total amount of salt + SC (by weight), with the salt concentration being 1 M. The volume ratio of solvent/aqueous phase was 9:1. The two-phase mixture was emulsified by placing it in an untrasound water bath (Bransonic Ultrasonics, Model 3210R-DTH) for 2–3 min, after which the emulsion was frozen in place by keeping the sample in liquid nitrogen for 10 min. The frozen sample was then lyophilized for a period of 36 h at 8 μm of Hg and −50°C.

Analytical

Samples in methanol were analyzed by HPLC, and samples in toluene or hexane were analyzed by GC. The HPLC method used a C18 column (Shimadzu Premier C18, 5 μ) with acetonitrile–water mixture as mobile phase with gradient from 70 to 100% acetonitrile and UV detection at 280 nm. The GC method consisted of a 0.53 mm×15 m DB5 column, with helium as the carrier gas, injector and detector temperatures of 250°C, and an oven temperature of 210°C.

Results and Discussion

Effect of Chemical Modification and Surfactants

Activity of Salt–Enzyme Preparations in Toluene and Hexane

Enzyme assays were conducted with native SC and PEG–SC to determine the effect of the PEG modification and addition of surfactant. The initial rate of transesterification was obtained by using the initial linear portion of the product formation curve. As indicated earlier, the data points (between 20 and 240 min) produced a linear plot, with an R^2 between 0.95 and 0.99. The results (Table 1) indicate that surfactants decreased rate of APEE transesterification in hexane for the native enzyme lyophilized with 99% salt; however, an enhancement of rate was observed in toluene. AOT has been shown to improve the rate of enzymatic reactions in certain organic solvents with pure enzymes and not in the presence of salts [19–21]. AOT can form either a micelle around the enzyme (in the presence of water) or can directly complex the enzyme in neat organic solvents. This is the first study, to our knowledge, investigating the effect of chemical modification and surfactants on salt-lyophilized enzymes. The idea behind using surfactants was to enable complexation of the salt–enzyme particle by the surfactant in the form of a reverse micelle to improve interaction with the substrate present in the solvent phase. Alternately, the substrate APEE itself may complex with the surfactant and improve its partitioning into the salt–enzyme phase, similar to a desolvation effect. Whereas the data suggest a beneficial effect of surfactant and chemical modification, the mechanism of rate enhancement is not completely clear.

Table 1 Initial rate of transesterification (V_{max}, mM/g SC-min) of APEE in hexane and toluene in presence of different surfactants.

	Hexane		Toluene	
	Native SC	PEG–SC	Native SC	PEG–SC
None	310	480	37	45
AOT	190	470	68	100
T 20	330	420	35	93

The estimated total error in calculation of the rates is 23% (maximum). This error is cumulative error in measurement of product concentration by GC and determination of enzyme concentration during weighing and lyophilization procedure.

The presence of AOT resulted in an about twofold increase in initial rate of conversion in toluene, whereas T 20 did not improve activity. A similar effect was also observed with PEG-modified SC (PEG–SC). The PEG attachment by itself did not increase the rate of reaction significantly, although a larger enhancement was observed with the surfactants. In the case of T 20, PEG modification resulted in a twofold increase in activity, not observed with the native enzyme. With hexane as solvent, the PEG modification resulted in a slight increase in activity compared to the activity of the native enzyme. However, given the 23% estimated error (Table 1) in determination of the rate, this difference is not significant. Whereas the surfactant AOT appeared to negatively affect the activity in hexane for the native enzyme, the PEG modification appeared to reduce this deleterious effect.

Activity of Salt–Enzyme Preparations in Methanol

The activity of the native and modified SC was also studied for the reaction: methanolysis of APEE, using the substrate, methanol, itself as a solvent. This was done for three different salt–enzyme preparations, consisting of 99% salt, 50% salt, and no added salt (except that present in buffer). It was found that the PEG modification resulted in a six- to sevenfold increase in the initial rates of conversion for the cases with no surfactant and with T 20 (Table 2). AOT was found to reduce the activity of the enzyme preparations in all experiments conducted in the solvent methanol. Previous reports on enzymatic conversion in organic solvents have shown the effect of the solvent dielectric constant on enzyme activity for salt-free enzymes [22]. Relationship between activity of AOT and PEG-modified SC to the hydrophobicity coefficient of various solvents has also been studied [20], however, only for the enzymes without salt-lyophilization. The decrease in activity of enzymes in organic solvents is attributed to the decreased water availability in organic media. Additionally, as the dielectric constant increases, the potential for removal of the

Table 2 Initial rates of conversion (V_{max}, mM/g SC-min) of APEE to APME in methanol.

	99% salt			50% salt			No salt (except buffer salts)		
	None	AOT	T 20	None	AOT	T 20	None	AOT	T 20
Native SC	370	330	220	26	15	34	45	20	37
PEG–SC	2420	750	2880	52	21	54	25	NA	25
PEG-C14-SC	420	250	410	7	NA	7	5.5	0.2	4.8

The estimated total error in calculation of the rates is 23% (maximum), as described in Table 1.

Table 3 APEE transesterification in millimolars per gram SC-min using SSENDs catalyst in hexane and comparison with reactions with native SC in presence of surfactants added postlyophilization.

	AOT	T 20
SC–AOT	1,050±330	
SC–T 20		730±80
Native SC	380	450

Note that the AOT and T 20 were not readded in the reactions with SSENDs catalyst (but they were present in the preparation due to addition before lyophilization). The surfactants indicated below were added (postlyophilization) only in the experiment with the native SC. The standard deviation for the SSEND samples is for duplicate experiments.

layer of hydration from enzyme microenvironment increases. The inclusion of salts with the enzymes, in essence, reduces the effect of solvents on the enzyme microenvironment and on the hydration layer, thereby increasing activity of the enzymes in organic media. A relationship between solvent parameters and salt-lyophilized enzymes has, however, not been reported. From our results, the addition of surfactants or chemical modification of enzymes appears to introduce additional complexity into the interaction between the enzyme and the solvent, further complicating any relationship that can be derived. A comparison of the enhancement in rate observed with 98 and 50% salt is not linear and resembles what has been reported with preparations without use of surfactants [23]. Further work is necessary to understand the enhancement observed in the rate of transesterification reactions. This is being pursued by systematically deriving catalytic efficiencies for various solvents in the presence and absence of surfactants and chemical modifications.

Surfactant-Assisted Salt Enzyme Nanodispersions

To increase dispersion or surface area of salt–enzyme preparations in organic media, a method of preparing surfactant-assisted salt enzyme nanodispersions (SSENDs) was explored. The goal was to create a fine dispersion of the salt enzyme lyophilized particles with the use of a surfactant prior to lyophilization by creation of a fine emulsion, followed by removal of solvent and water by lyophilization. Microscopic observation of the emulsion indicated a submicron dispersion of the aqueous phase in the solvent. The results of the enzyme assay using SSENDs catalyst is shown in Table 3. This experiment was conducted in hexane to determine if such a preparation can enhance activity of the enzyme because postlyophilization addition of the surfactant did not improve the rate of the reaction. The results show that a two- to threefold higher rate can be obtained by preparation of the salt–SC using the SSENDs preparation method.

Table 4 Acylation of bergenin by native and chemically modified SC in hexane and toluene.

	Bergenin 4,11-diacetate	Bergenin 11-acetate
Native SC	20	5
PEG–SC	20	6
PEG-C14-SC	18	5

Rate is given in millimolars per gram SC-min. The response factor for bergenin was used for quantitation of the products.

Derivatization of Bergenin

One of the goals of this study was to identify experimental conditions for biotransformations in organic media and apply them to derivatization of drug candidates. Along these lines, derivatization of bergenin was studied using chemically modified SC to determine change in the rate of reaction. Bergenin consists of two hydroxyl groups capable of acylation by lipase biocatalysis, at positions 4 and 11 [18]. Thus, alteration of specificity due to introduction chemical modification was also investigated. The results (Table 4) indicate that the chemical modification did not introduce any significant change in specificity or rate of the reaction.

Conclusions

Effect of surfactants and chemical modification was studied on salt-lyophilized SC in organic solvents. Modification with PEG was found to enhance the initial rate of transesterification marginally in toluene but about five- to sixfold in methanol. Addition of AOT to the reaction mixture was found to enhance the rate by about threefold in toluene, but reduced it in hexane and methanol. The combined effect of the presence of salt, surfactant, and solvent appears to result in a complex interaction with the enzyme resulting in an, as yet, unpredictable change in the reaction rate as a result of a change in its microenvironment. Further work to determine catalytic efficiencies for a variety of solvents and surfactants is necessary to better understand the interactions. Creation of fine emulsions of enzyme–salt solutions prior to lyophilization using surfactants appears to enhance the activity of lyophilized biocatalysts prepared using the SSENDs method. Finally, conversion of bergenin in toluene was not affected significantly by chemical modification of the enzyme. Further work investigating the effect of surfactants on bergenin conversion is under way.

Overall, chemical modification and surfactants appear to offer moderate enhancements in some cases compared to salt-lyophilized enzymes. However, these enhancements are small compared to the original advantages of salt lyophilization.

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References

1. Altreuter, D. H., & Clark, D. S. (1999). *Current Opinion in Biotechnology, 10*, 130–136.
2. Michels, P. C., Khmelnitsky, Y. L., Dordick, J. S., & Clark, D. S. (1998). *Trends in Biotechnology, 16*, 210–215.
3. Rich, J. O., Michels, P. C., & Khmelnitsky, Y. L. (2002). *Current Opinion in Chemical Biology, 6*, 161–167.
4. Dordick, J. S. (1992). *Biotechnology Progress, 8*, 259–267.
5. Adamczak, M., & Krishna, S. H. (2004). *Food Technology and Biotechnology, 42*, 251–264.
6. Gupta, M. N., & Roy, I. (2004). *European Journal of Biochemistry, 271*, 2575–2583.
7. Lee, M. Y., & Dordick, J. S. (2002). *Current Opinion in Biotechnology, 13*, 376–384.
8. D'Souza, S. F. (1999). *Current Science, 77*, 69–79.
9. Dai, L. Z., & Klibanov, A. M. (1999). *Proceedings of the National Academy of Sciences of the United States of America, 96*, 9475–9478.

10. Morgan, J. A., & Clark, D. S. (2004). *Biotechnology and Bioengineering, 85*, 456–459.
11. Sawae, H., Sakoguchi, A., Nakashio, F., & Goto, M. (2002). *Journal of Chemical Engineering of Japan, 35*, 677–680.
12. Song, B. D., Ding, H., Wu, J. C., Hayashi, Y., Talukder, M., & Wang, S. C. (2003). *Chinese Journal of Chemical Engineering, 11*, 601–603.
13. Mine, Y., Fukunaga, K., Yoshimoto, M., Nakao, K., & Sugimura, Y. (2001). *Journal of Bioscience and Bioengineering, 92*, 539–543.
14. Wang, L. F., Zhu, G. Y., Wang, P., & Newby, B. M. Z. (2005). *Biotechnology Progress, 21*, 1321–1328.
15. Hudson, E. P., Eppler, R. K., & Clark, D. S. (2005). *Current Opinion in Biotechnology, 16*, 637–643.
16. Altreuter, D. H., Dordick, J. S., & Clark, D. S. (2003). *Biotechnology and Bioengineering, 81*, 809–817.
17. Prior, R. L., & Cao, G. (1999). *Proceedings of the Society for Experimental Biology and Medicine, 220*, 255–261.
18. Mozhaev, V. V., Budde, C. L., Rich, J. O., Usyatinsky, A. Y., Michels, P. C., Khmelnitsky, Y. L., et al. (1998). *Tetrahedron, 54*, 3971–3982.
19. Bindhu, L. V., & Abraham, T. E. (2003). *Biochemical Engineering Journal, 15*, 47–57.
20. Kwon, O. H., Imanishi, Y., & Ito, Y. (1999). *Biotechnology and Bioengineering, 66*, 265–270.
21. Paradkar, V. M., & Dordick, J. S. (1994). *Biotechnology and Bioengineering, 43*, 529–540.
22. Clark, D. S. (2004). *Philosophical Transactions of the Royal Society of London Series B, Biological Sciences, 359*, 1299–1307.
23. Ru, M. T., Wu, K. C., Lindsay, J. P., Dordick, J. S., Reimer, J. A., & Clark, D. S. (2001). *Biotechnology and Bioengineering, 75*, 187–196.