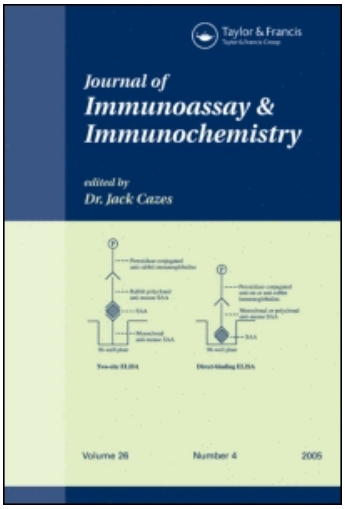

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ABSTRACT

The influence of various additives, such as organic solvents, polyhydric alcohols, salts, polymers, and cross-linker, on the stability and storage ability of penicillinase-morphine conjugate was studied in liquid and solid (freeze dried) states. The results of these experiments showed that using low concentrations of $CaCl_2$ (0.1–0.2%) could stabilize enzyme activity in both states for more than seven months. The immunoreactivity of antigen toward the antibody did not change significantly. However, a cross-linker such as glutaraldehyde and various additives such as dimethylsulfoxide, glycerol, polyethylene glycol, gelatin, dextran, ammonium sulfate, lactose, and sucrose did not have any effect on stability.

In addition, it was found that the presence of lactose and sucrose in the lyophilization procedure gives a significant

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amount of protection to the enzyme, which could last for a period of seven months and preserve almost 95% of the enzyme activity, as well as immunoreactivity of the tracer molecule.

Key Words: Stabilization; Enzyme immunoassay; Penicillinase

INTRODUCTION

In immunoassay procedures used in clinical diagnosis, antibodies and antigens are conjugated with enzymes.[1,2] One of the most important problems, with which the development of enzyme immunoassays is confronted, is reagent stability.[3] In an assay procedure, the enzyme conjugate compositions are usually prepared well in advance of the time the assay itself is initialized. Due to the instability of the enzyme, the storage of these enzyme conjugate compositions can lead to loss of enzyme activity over the time. This instability can be a significant disadvantage, because shipping, distribution to customers, and storage in the inventory usually involve substantial time delays between preparation of enzyme conjugate and their actual use in the laboratory. Furthermore, the enzyme conjugates can be subjected to a wide temperature variation or other conditions promoting loss of enzyme activity. Accordingly, using the enzyme conjugate compositions, which exhibit dramatically increased stability, may be a great improvement in the assay field.

Medium and protein engineering are two strategies being mainly applied in enzyme stabilization technology. Medium modification involves the use of additives such as ions,[4,5] polymers,[6,7] solvents,[8,9] and sugars.[10,11] This type of modification, being easy to perform, is most commonly used, while protein engineering, which involves chemical modification and genetic engineering of the protein, is seldom used because the mechanisms are not known properly.[12]

The aim of this study is to investigate the stability of penicillinase-morphine conjugate in liquid and solid states, in the presence of a chemical modifier and a number of known stabilizers.

EXPERIMENTAL

Penicillinase (β-lactamase EC, 3.5.2.6), calcium chloride, ammonium sulfate, dimethylsulfoxide (DMSO), polyethylene glycol 8000 (PEG),

dextran T-70, gelatin, glycerol, lactose, sucrose, glutaraldehyde, 1,ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC), *N*-hydroxy succinimide, dimethyl formamide (DMF), bovine serum albumin (BSA) and penicillin V were obtained from Sigma Chemical Company, St. Louis, MO, USA.

Preparation of Penicillinase-Morphine Conjugate

Morphine derivative was conjugated to penicillinase following the method of activated ester.[13] Six hundred milligram of morphine-3 hemisuccinate (morphine-3HS) was dissolved in 150 μL of DMF, and then *N*-hydroxy succinimide (450 μg) and EDC (100 μg) were added to the above solution and incubated at room temperature (1 h) while stirring. Penicillinase (8 mg, equivalent to 4 mg protein) dissolved in 500 μL of phosphate buffer saline (PBS) (0.01 molar, pH 7.2) and 100 μL of pyridine was added to the above solution, drop by drop, within 20 min, while stirring. The final solution was incubated at 4°C for 16 h. At the end of incubation time, the product was chromatographed on a Sephadex G25 column, and stored as explained elsewhere.[14]

Checkerboard Titration Assay

Different concentrations of anti-morphine-6-hemisuccinate (anti-morphine-6HS) (4, 2, 1 and 0.5 μg/well) were prepared in PBS buffer, and coated onto wells of a microtiter plate in 8 replicates. One row was coated with normal rabbit serum (1 : 300), to represent a non-specific binding (NSB) index. Wells were blocked, washed and added with different dilutions of conjugate (1 : 100, 1 : 200, 1 : 400, 1 : 800 and 1 : 1600) containing no stabilizer, incubated for 45 min at 37°C, washed and added with 100 μL freshly prepared substrate solution (3.1 g penicillin V in 10 mL of 0.2 M PBS, pH 7.4). Wells were then incubated at 37°C for 1 h, added with 150 μL of starch iodine solution of which preparation has been reported elsewhere,[14] incubated for 10 min at room temperature, and measured at 600 nm by ELISA reader.

Standard Curve Preparation

Different dilutions (10 pg, 100 pg, 1000 pg, 10 000 pg and 100 000 pg/50 μL) of morphine sulfate were prepared from a stock buffer

solution (1 mg/mL) and added to pre-antibody coated wells (2 μg/well) which were blocked as explained before, incubated for 1 h and added with 50 μL of enzyme conjugate (1 : 200) and incubated for another 45 min at 37°C. Wells were drained, washed, and added with substrate solution as explained before. The rest of experiments were performed as explained above. A standard curve was constructed as a function of percent binding vs. concentration of morphine.

Thermostability of Enzyme in the Presence of $CaCl_2$

In order to obtain a proper concentration of $CaCl_2$ to be used as stabilizer, penicillinase (250 μg) was diluted in PBS (25 mL) and divided into a number of tubes containing 0, 0.12, 0.25, 0.5 and 1% of $CaCl_2$. The solutions were kept at 40 and 50°C for 50 h. The enzyme activity was measured every 10 h (including zero hour). To investigate the effect of heat on the stability of enzyme-hapten conjugate (enzyme activity and immunoreactivity), a known concentration of conjugate was selected and treated with 0, 0.12, 0.25, 0.5, and 1% of $CaCl_2$ and kept at 40°C for 50 h. The stability was checked each 10 h (including zero hour) by adding 50 μL of the sample into the wells of a microtiter plate previously coated with 2 μg of purified anti-morphine antibody and blocked with 0.5% gelatin. The rest of experiments were performed as explained before.

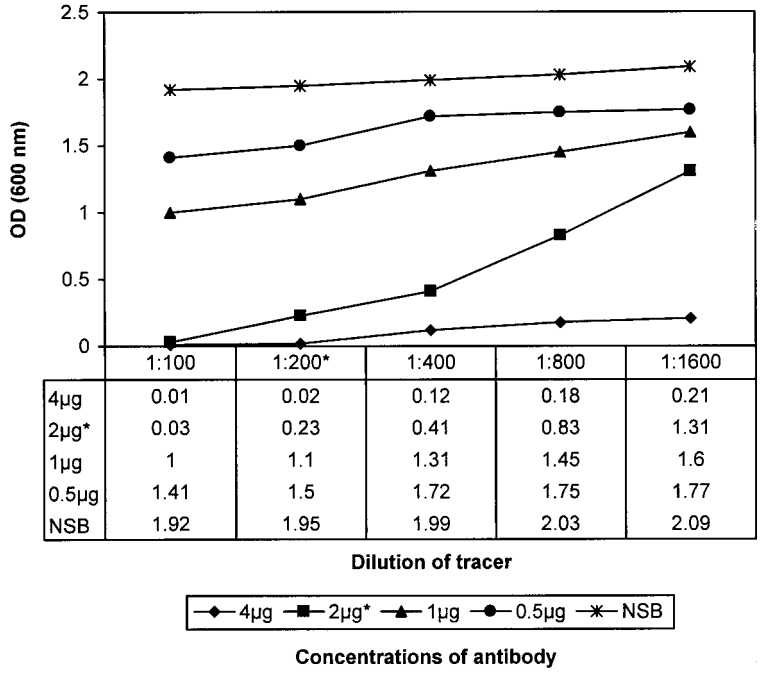
Optimum Concentration of Stabilizers Using Checkerboard Titration Assay

In order to obtain the best concentration of various stabilizers and to study their effect on the immunoreactivity of antigen-antibody reaction, concentrations of $CaCl_2$ (0.2%), ammonium sulfate (0.2%), PEG (2%), dextran (1%), gelatin (0.5%), DMSO (40, 20 and 10%), glycerol (40 and 20%), and glutaraldehyde (3 nmol) were added to a fixed concentration of enzyme conjugate (1 : 200). The checkerboard titration assay was performed as explained before. The proper concentration for each stabilizer was selected, (i.e., the concentration of stabilizer showing minimum alternation on enzyme activity and immunoreactivity, as compared to the conjugate solution containing no stabilizer). The enzyme stored at 4°C in liquid condition (final concentration) and 4°C in lyophilized form (containing 0.03 BSA used as bulking agent). The stock conjugate solution, without stabilizer, was also aliquoted and stored at previously explained thermal

and physical conditions. ELISAs were performed at the end of each month for seven successive months.

RESULTS

Figure 1 shows the titration assay of conjugate prepared in our experiments. The best antibody titer and the dilution of enzyme conjugate were found to be 2 μg/well and 1 : 200, respectively. Figure 2 denotes a typical standard graph in which the enzyme conjugate is used without stabilizers. The sensitivity is found to be from 10 pg up to 100 ng/well, and the slope of this curve was calculated to be −1. Figures 3 and 4 show the effect of temperature (40 and 50°C) on enzyme activity in the presence of various concentrations of $CaCl_2$ at different times. The results revealed that the low concentrations of $CaCl_2$ (0.12–0.25) had a better effect on preservation of enzyme activity. Figure 5 shows our results when the stability of conjugate



	1:100	1:200*	1:400	1:800	1:1600
4μg	0.01	0.02	0.12	0.18	0.21
2μg*	0.03	0.23	0.41	0.83	1.31
1μg	1	1.1	1.31	1.45	1.6
0.5μg	1.41	1.5	1.72	1.75	1.77
NSB	1.92	1.95	1.99	2.03	2.09

Figure 1. Checkerboard titration assay of morphine-3HS-penicillinase. *Concentrations of antibody and tracer molecule used throughout the experiments.

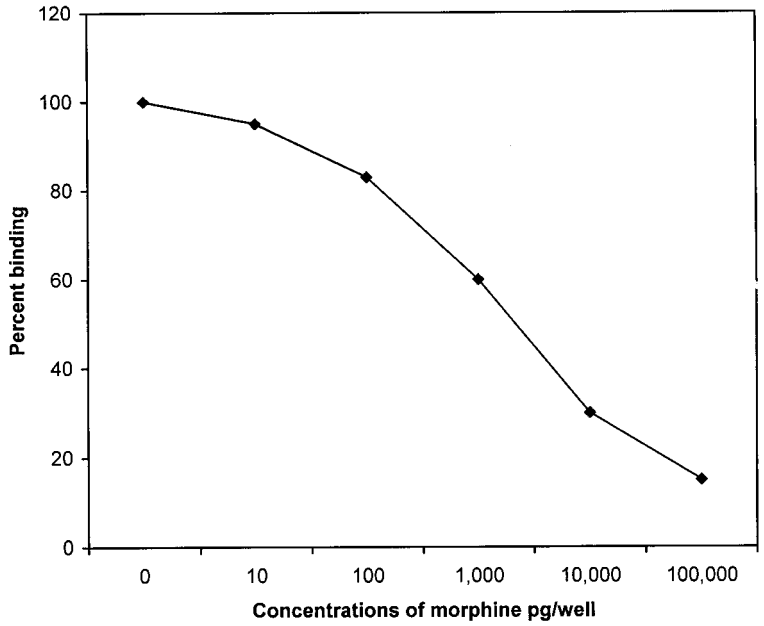


Figure 2. Standard graph of morphine measurement ($tg\alpha = -1$). *Each point is the result of four experiments with a coefficient of variation (CV) calculated to be within 5%.

was studied for up to 50 h at 40°C. Here again the results indicated that the low concentrations of $CaCl_2$ (0.12–0.25%) responded better in term of enzyme activity and immunoreactivity.

Table 1 denotes the final concentration of stabilizers used in our experiments. A cutoff value of around 0.2 optical density (OD) was selected as the best OD, accordingly, for a concentration of stabilizers used throughout these experiments. Tables 2 and 3 represent our results on the stability measurement after seven months in liquid and solid forms (lyophilized). Here, the results again indicated that 0.2% $CaCl_2$ could well preserve almost 95% of enzyme activity and immunoreactivity after seven months of storage throughout different doses of morphine in standard curve in both forms (liquid and lyophilized). For an enzyme stock solution kept at 4°C, the slope of the standard curve in the case of $CaCl_2$, in both conditions, were calculated to be −0.68 and −0.68 at zero time and −0.75 and −0.8 after seven months in liquid and solid states, respectively, for both times. However, it will be easy to compare the stability effect of additives when only zero dose ODs are considered.

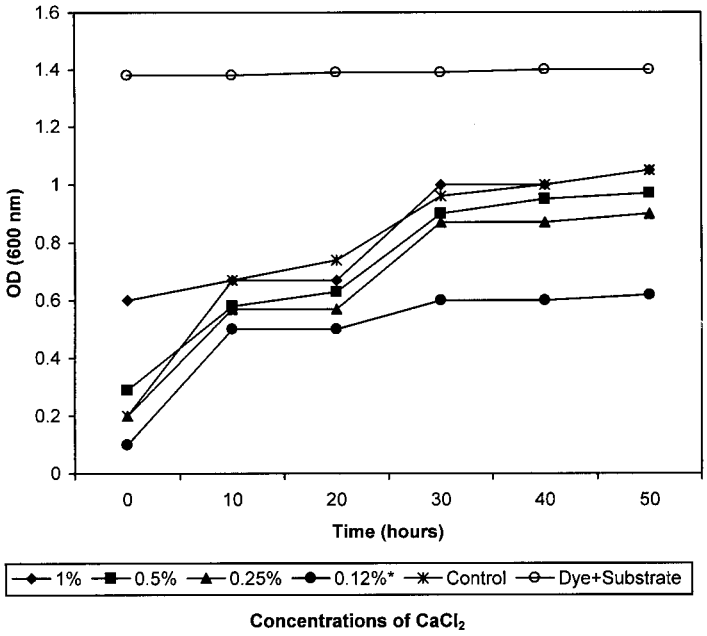


Figure 3. Effect of temperature (40°C) on penicillinase activity in the presence of different concentrations of $CaCl_2$. *Highest enzyme activity corresponding to lowest optical density at a concentration of 0.12% $CaCl_2$.

DISCUSSION

Penicillinase as an enzyme used in biosensor technology[15] and immunoassay[16–18] needs to be stabilized in both solid and liquid states. The examples available in the literature show that stability may result from modification in the microenvironment of the enzymes. The enzyme activity was found to be modulated by using salts,[4,5] organic solvents,[8,9] polymers,[6,7] and sugars.[10,11] In this paper, the stability of penicillinase-morphine conjugate, in the presence of various additives such as $CaCl_2$, $(NH_4)_2SO_4$, sucrose, lactose, PEG, dextran, gelatin, DMSO, glycerol, and glutaraldehyde, was studied for immunoassay purposes in both liquid state and lyophilized form in buffer solution with a pH of 7.2. Primarily, thermostability of enzyme was performed using different concentrations of $CaCl_2$ in various time elapses. The results clearly indicated that the low concentration of $CaCl_2$ provided much higher stability, while increasing the concentration caused destabilization, which resulted in total destruction of enzyme activity

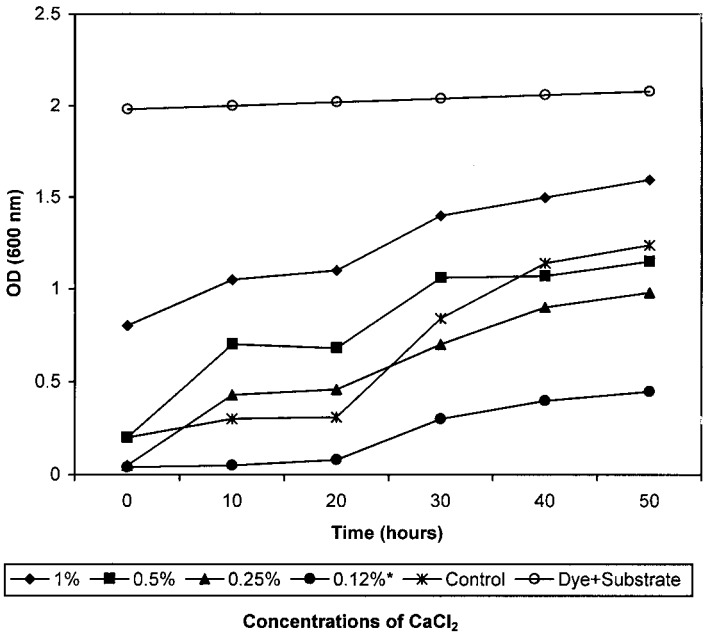


Figure 4. Effect of temperature (50°C) on penicillinase activity in the presence of different concentrations of $CaCl_2$. *Highest enzyme activity corresponding to lowest optical density at a concentration of 0.12% $CaCl_2$.

at a concentration of about 1% $CaCl_2$. These were in agreement with reports in the literature where a low concentration of salt ($CaCl_2$) was recommended for other enzymes.[7] It is known that refolding of the protein should be attempted under conditions that favour the strengthening of some kinds of noncovalent interactions such as hydrophobic ones, which could be achieved by a salt concentration altering water structure and thereby enhancing hydrophobic interactions.[5] The results shown here may also explain the possibility of calcium functioning as a bridging factor within the polypeptide chain, which reduces the flexibility of the polypeptide backbone, thus enhancing stability. In the case of penicillinase, however, the same is not observed for another common salt (ammonium sulfate) used for general stabilization of a number of other common enzymes.[7]

When comparing between the effect of additives (Tables 2 and 3), it was generally found that, at the concentrations used in this study (except $CaCl_2$), they did not have a considerable stabilizing effect on penicillinase, while this was not the case for other enzymes such as lactate and malate dehydrogenase.[6] In case of glutaraldehyde, although, it was already

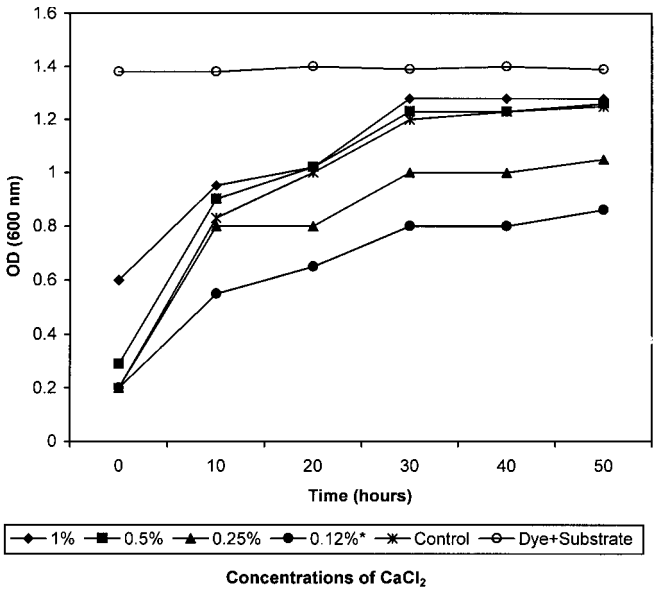


Figure 5. Effect of temperature (40°C) on penicillinase-morphine activity in the presence of different concentrations of $CaCl_2$. *Highest enzyme activity corresponding to lowest optical density at a concentration of 0.12% $CaCl_2$.

Table 1. Results of Checkerboard Titration Assay Using 2 μg/well Anti-Morphine-6HS-BSA and 1 : 200 of Enzyme Conjugate (Morphine-3HS-Pen) in the Presence of Various Concentrations of Stabilizers

Stabilizers	Concentrations	OD at Zero Dose and Zero Time
$CaCl_2$	0.2%	0.04
$(NH_4)_2SO_4$	0.2%	0.12
Lactose	3%	0.22
Sucrose	3%	0.14
PEG	2%	0.21
Dextran	1%	0.06
Gelatin	0.5%	0.25
DMSO	10%*, 20%, 40%	0.28**, 0.5, 0.6
Glycerol	20%*, 40%	0.3**, 0.45
Glutaraldehyde	3 nmol	0.5

*Suitable concentration of stabilizer and **their corresponding OD.

Table 2. Effect of Different Stabilizers on Enzyme Activity and Immunoreactivity at Zero Time and After 7 Months of Storage in Liquid State

		OD at Different Concentrations of Morphine (pg/well)							
Stabilizers	Time (Month)	Total	NSB	0	10	10^2	10^3	10^4	10^5
Control	Zero	2.5	1.99	0.25	0.38	0.61	0.91	1.21	1.45
	7th	2.7	2	1.01	1.16	1.25	1.39	1.69	1.79
$CaCl_2$	Zero*	2.5	1.99	0.09	0.19	0.29	0.69	0.91	1.07
	7th*	2.7	2	0.27	0.51	0.66	0.95	1.20	1.38
$(NH_4)_2SO_4$	Zero	2.5	1.99	0.09	0.19	0.29	0.69	0.92	1.08
	7th	2.7	2	1.03	1.15	1.24	1.37	1.50	1.69
Lactose	Zero	2.5	1.99	0.12	0.25	0.38	0.56	0.81	1.13
	7th	2.7	2	0.81	0.95	1.11	1.30	1.42	1.55
Sucrose	Zero	2.5	1.99	0.12	0.34	0.49	0.78	0.98	1.11
	7th	2.7	2	1.02	1.15	1.25	1.48	1.65	1.79
PEG	Zero	2.5	1.99	0.16	0.29	0.41	0.71	0.99	1.21
	7th	2.7	2	1.25	1.43	1.55	1.63	1.81	1.88
Dextran	Zero	2.5	1.99	0.08	0.19	0.31	0.71	0.99	1.19
	7th	2.7	2	0.89	0.99	1.22	1.35	1.49	1.70
Gelatin	Zero	2.5	1.99	0.15	0.29	0.41	0.75	1.05	1.26
	7th	2.7	2	1.05	1.19	1.38	1.49	1.55	1.67
DMSO	Zero	2.5	1.99	0.21	0.35	0.61	0.87	1.11	1.31
	7th	2.7	2	1.04	1.16	1.37	1.51	1.63	1.72
Glycerol	Zero	2.5	1.99	0.25	0.41	0.55	0.75	0.98	1.28
	7th	2.7	2	1.16	1.29	1.39	1.52	1.68	1.79
Glutaraldehyde	Zero	2.5	1.99	0.51	0.98	1.22	1.48	1.61	1.82
	7th	2.7	2	1.96	1.98	1.98	1.98	1.99	1.99

*OD at zero time and after 7 months at a 0.12% concentration of $CaCl_2$ in different doses of morphine.
Slope of standard curve at zero time = −0.68, slope of standard curve after 7 months = −0.75.

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Table 3. Effect of Different Stabilizers on Enzyme Activity and Immunoreactivity at Zero Time and After 7 Months of Storage in Solid State

		OD at Different Concentrations of Morphine (pg/well)							
Stabilizers	Time (Month)	Total	NSB	0	10	10^2	10^3	10^4	10^5
Control	Zero	2.5	1.99	0.15	0.27	0.36	0.68	0.93	1.28
	7th	2.7	2	0.89	1.06	1.21	1.31	1.56	1.69
$CaCl_2$	Zero*	2.5	1.99	0.12	0.21	0.33	0.79	0.99	1.08
	7th*	2.7	2	0.21	0.31	0.42	0.95	1.18	1.38
$(NH_4)_2SO_4$	Zero	2.5	1.99	0.11	0.21	0.33	0.79	0.93	1.05
	7th	2.7	2	0.71	0.88	0.95	1.07	1.42	1.61
Lactose	Zero	2.5	1.99	0.12	0.26	0.38	0.54	0.78	1.08
	7th	2.7	2	0.13	0.22	0.39	0.67	0.92	1.31
Sucrose	Zero	2.5	1.99	0.18	0.29	0.41	0.65	0.75	1.05
	7th	2.7	2	0.25	0.31	0.52	0.75	1.02	1.31
PEG	Zero	2.5	1.99	0.29	0.45	0.68	0.99	1.29	1.39
	7th	2.7	2	1.41	1.58	1.68	1.71	1.96	1.97
Dextran	Zero	2.5	1.99	0.17	0.28	0.51	0.79	0.99	1.26
	7th	2.7	2	1.18	1.28	1.36	1.41	1.52	1.75
Gelatin	Zero	2.5	1.99	0.25	0.38	0.59	0.81	0.99	1.28
	7th	2.7	2	1.26	1.49	1.65	1.75	1.81	1.89
DMSO	Zero	2.5	1.99	0.55	0.89	1.22	1.48	1.69	1.78
	7th	2.7	2	1.14	1.57	1.69	1.79	1.83	1.95
Glycerol	Zero	2.5	1.99	0.43	0.65	0.89	0.99	1.19	1.33
	7th	2.7	2	1.71	1.89	1.89	1.95	1.95	1.99
Glutaraldehyde	Zero	2.5	1.99	0.81	1.39	1.75	1.85	1.86	1.89
	7th	2.7	2	1.95	1.97	1.98	1.99	1.99	1.99

*OD at zero time and after 7 months at a 0.12% concentration of $CaCl_2$ in different doses of morphine.
Slope of standard curve at zero time = −0.68, slope of standard curve after 7 months = −0.8.

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reported that this cross-linker may be used for penicillinase stabilization;[15] our results, however, showed that the enzyme activity was completely lost even at a very low concentration of glutaraldehyde (3 nmol) after one month of storage (data not shown). This may be due to introduction of intramolecular cross-linking, which may destroy the active site of the enzyme molecule. The same situation was observed for DMSO and glycerol, which acted better at low concentrations. It is further denoted that, although the use of various polymers such as polyvinyl alcohol (PVA) and polyethylene oxide (PEO), had a stabilizing effect on enzyme horseradish peroxidase (HRP)[3] and gelatin had a stabilizing effect on lactate dehydrogenase.[6] However, in our study, none of the polymers could preserve the enzyme activity during the study period, but short stabilization for three months at 4°C was observed (data not shown), which may explain the existence of successful reports in the literature.

In order to understand the effect of freeze-drying in the presence or absence of stabilizers, the additives were added in the same concentration as that of liquid and freeze-dried states. The results satisfied the assumption that disaccharide molecules form hydrogen bound with protein when water is removed, which results in maintaining the tertiary protein structure and forming a glassy structure, and consequently leads to extremely low molecular motion determining the stabilization of biological material. In addition to additives, other factors such as pH (7.2), buffer concentration (10 mmol, PBS), moisture content (5%), protein concentration and bulking agent (BSA), which affect the quality of freeze-dried products, were carefully maintained.[19] On this basis, the presence of lactose and sucrose in comparison to other stabilizers that were used in these experiments (except $CaCl_2$) appeared to be working properly and effectively. Based on the observation that drying in the absence of disaccharide caused a dramatic loss of enzyme activity, we hence concluded that use of disaccharides would apparently increase the storage ability of the enzyme in freeze-dried condition.

In summary, the data obtained in this work clearly showed that $CaCl_2$ at a concentration of about 0.2% could prevent destabilization of enzyme conjugate (penicillinase) for long term storage. Also it was revealed that freeze-drying would generally postpone the destructive effect of long storage on enzyme activity, especially when disaccharides such as sucrose and lactose were used at a proper concentration.

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