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STORAGE STABILITY OF WATER-INSOLUBLE ENZYMES COVALENTLY COUPLED TO ORGANIC AND INORGANIC CARRIERS

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SUMMARY

1. Water-insoluble enzymes prepared by covalent coupling to organic polymers have been compared for storage stability under various conditions with enzymes insolubilized by covalent coupling to inorganic carriers including glass, colloidal silica, alumina, and hydroxyapatite.

2. Enzymes covalently coupled to inorganic carriers were found to have greater stability than enzymes covalently attached to organic polymers when stored for several weeks at 4° and 23° dry or in distilled water.

3. Enzymes covalently coupled to the inorganic carriers by sulfonamide linkage were not as stable during storage as enzymes coupled by azo linkage.

INTRODUCTION

Enzymes have been insolubilized by covalent attachment to various organic polymers including cellulose[1–3], polyaminopolystyrene, and polyaminoacids[4–6]. Enzymes have also been insolubilized by copolymerization[4,5] and gel entrapment[7,8].

I have recently reported the covalent attachment of enzymes to inorganic carriers[9–11]. These enzymes have great temperature and operational stability when compared to similar enzymes covalently coupled to organic polymers.

One of the major reasons for insolubilizing enzymes is to permit the reuse of the materials, particularly if the enzyme is difficult to obtain or is expensive to purchase. For an enzyme derivative to be reuseable in practice, one must be able to store the material for a reasonable length of time after using the derivative.

This study was designed to compare the storage stability of several enzymes covalently coupled to organic and inorganic carriers under various storage conditions.

METHODS AND MATERIALS

Enzymes coupled to organic carriers

Enzyme derivatives of trypsin, ficin, papain and glucose oxidase covalently coupled to cellulose by the azide method[12] were obtained commercially. A derivative of trypsin obtained by coupling the enzyme with a copolymer of maleic anhydride and ethylene[4,5], and a papain derivative coupled to a polyaminoacid by azo linkage[13] were also purchased from commercial sources.

Enzymes coupled to inorganic carriers

The enzymes trypsin, ficin, papain, and glucose oxidase were covalently coupled to 96% porous silica glass, pore size 735 ± 50 Å (refs. 9–11).

The porous glass was first refluxed overnight in a 10% solution of γ-aminopropyltriethoxysilane in toluene, washed with toluene and dried. The isothiocyanate derivative was prepared by refluxing the alkylamine glass derivative overnight in a 10% solution of thiophosgene in chloroform. The glass was then washed with chloroform, air-dried and immediately coupled to a 0.25% solution of enzyme at pH 9.0 in carbonate buffer at room temperature.

The arylamine derivative was prepared by reacting the alkylamine glass with *p*-nitrobenzoyl chloride in chloroform containing 5% triethylamine. The nitro groups were reduced in boiling aqueous sodium dithionite. The arylamine glass was then diazotized and coupled to a 0.25% solution of enzyme at 0°.

In addition, glucose oxidase was covalently coupled to alumina and hydroxyapatite by the same coupling technique used with porous glass. A sample of trypsin was also covalently coupled to colloidal silica.

Insoluble enzyme derivatives examined

All CGW samples were reacted for 2–3 h unless otherwise stated.

CGW-AT. Prepared from crystalline trypsin covalently coupled to porous glass by azo linkage.

CGW-ST. Prepared from crystalline trypsin covalently coupled to porous glass by sulfonamide linkage.

M(1:1). Prepared from a copolymer of maleic anhydride and ethylene to which crystalline trypsin was added in a 1:1 ratio.

M(1:4). Prepared same as above in a 1:4 ratio of enzyme to polymer.

M(4:1). Prepared same as above in a 4:1 ratio of enzyme to polymer.

Cell-T. Prepared by coupling crystalline trypsin to cellulose by the azide method.

CGW-Silica-ST. Crystalline trypsin covalently coupled to 4-μ-size silica particles by sulfonamide linkage.

CGW-AF. Ficin covalently coupled to porous glass by azo linkage.

CGW-SF. Ficin covalently coupled to porous glass by sulfonamide linkage.

Cell-F. Ficin covalently coupled to cellulose by the azide method.

CGW-AP1. Crude papain covalently coupled to porous glass by azo linkage.

CGW-AP8. Crude papain covalently coupled to porous glass by azo linkage. However, the initial enzyme solution concentration during coupling was 1% and coupling time was decreased to 10 min.

CGW-AP9. Crystalline papain covalently coupled to porous glass by azo linkage.

CGW-SP1. Crude papain covalently coupled to porous glass by sulfonamide linkage.

CGW-SP2. Crude papain covalently coupled to Code 7900, 96% silica (Corning Glass Works, Corning, New York) glass particles by sulfonamide linkage.

CGW-SP4. Crystalline papain covalently coupled to porous glass by sulfonamide linkage.

Cell-P. Crystalline papain covalently coupled to cellulose by the azide method.

M-P. Crystalline papain covalently coupled to a polyaminoacid by azo linkage.

CGW-AlGO. Glucose oxidase covalently coupled to alumina by sulfonamide linkage.

CGW-HyGO. Glucose oxidase covalently coupled to hydroxyapatite by sulfonamide linkage.

CGW-SGO. Glucose oxidase covalently coupled to porous glass by sulfonamide linkage.

Cell-AGO. Glucose oxidase covalently coupled to cellulose by the azide method.

Soluble GO. Crystalline glucose oxidase dissolved in distilled water.

Trypsin

Assay techniques. The assay method consisted of adding a given quantity of enzyme derivative in 1 ml of water to 4 ml of 1% heat-denatured Hammerstein casein dissolved in 0.1 M phosphate buffer (pH 7.0). The hydrolysis was allowed to continue in a shaker bath at 23° for 10 min, at which time 5.0 ml of 10% trichloroacetic acid was added. The trichloroacetic acid precipitate was allowed to stand for 20 min, filtered, and the filtrate measured spectrophotometrically at 280 mμ. Insolubilized enzyme activity was determined by using a standard curve of the free enzyme.

Ficin

Assay techniques. Samples of insolubilized enzyme were weighed into 10-ml vials and 1 ml of a solution containing cysteine and EDTA was added. The enzyme was allowed to stand 20 min to insure activation. At zero time, 4 ml of a 1% solution of heat-denatured casein in 0.1 M phosphate buffer (pH 6.2) were added. The reaction was continued for 60 min and stopped by addition of 5 ml of a 10% solution of trichloroacetic acid.

The precipitated material was allowed to stand 20 min and was then filtered and measured spectrophotometrically at 280 mμ.

Papain

Assay techniques. Same as for ficin.

Glucose oxidase

Assay techniques. To a given quantity of enzyme derivative was added 49 ml of 1% dextrose dissolved in 0.05 M phosphate buffer (pH 6.0). The reactants were stirred 5 min and a 25-ml aliquot immediately filtered. To the filtrate was added 0.5 ml of developer containing 100 μg of peroxidase per ml and 0.05% *o*-dianisidine per ml. The reaction color was allowed to develop for 2–3 min, stopped with 1 drop of

4 M HCl and the results determined spectrophotometrically at 460 mμ. The activity was determined against a standard curve of free enzyme.

Storage conditions

The enzymes covalently coupled to the organic carriers were received in a freeze-dried condition. They were stored at 4° and 23° as received and in distilled water at 4° and 23°.

The enzymes covalently coupled to the inorganic carriers were stored at 4° and 23° as removed from a Buchner funnel immediately after washing (still damp) and in distilled water at 4 and 23°. Storage ranged from 10 to 80 days. Enzymes were assayed before and after storage.

RESULTS AND DISCUSSION

Trypsin

The CGW trypsin derivatives prepared from porous glass covalently coupled to crystalline trypsin as previously described[9–11] were stored without freeze-drying at room temperature and at 4°.

The conditions of storage for the enzyme derivatives coupled to inorganic carriers were more adverse than for the commercial preparations since water was always present. Freeze-dried enzymes, generally, are more stable than when in water or under humid conditions. These storage conditions were chosen to give any benefit of doubt to the enzymes coupled to organic carriers.

The results given in Table I show that the CGW-ST was completely inactive by the 68th day.

TABLE I

SUMMARY OF STABILITY STUDIES ON TRYPSIN DERIVATIVES

Derivative	*Condition of storage*	*Original activity (mg/g)* ***	*Storage time (days)*	*Final activity (mg/g)* ***	*Percentage original activity remaining*
CGW-AT	5°	1.42	72	1.12	79
CGW-AT	23°	1.42	72	1.26	89
CGW-ST	5°	0.97	68	—	0
CGW-ST	23°	0.97	68	—	0
Cell-T*	5°	3.82	42	2.4	60
Cell-T	23°	3.82	63	2.4	60
M(1:1)**	5°	6.50	22	6.50	100
M(1:1)	23°	6.50	42	6.50	100
M(1:1)	5°, in water	6.50	30	—	0
M(1:4)	5°	26.0	63	10.3	48
M(1:4)	23°	26.0	36	22.2	84
M(4:1)	5°	41.0	63	8.1	19.5
M(4:1)	23°	41.0	63	5.3	12.9
CGW-Silica-ST	23°	1.32	42	0.757	57.6

* Cellulose derivative of trypsin.

** Represents weight of ratio of enzyme to copolymer of maleic anhydride and ethylene.

*** Expressed as mg of enzyme protein per g of derivative determined from the activity of the derivative assuming that the attached and free solution enzymes have identical activities.

TABLE II

SUMMARY OF STABILITY STUDIES OF FICIN DERIVATIVES

Derivative	*Condition of storage*	*Time in storage (days)*	*Original activity (mg/g)***	*Final activity (mg/g)***	*Percent activity retained*
CGW-AF	5°	60	1.06	1.06	100
CGW-AF	23°	60	1.06	1.06	100
CGW-SF	5°	60	4.20	4.20	100
CGW-SF	23°	60	4.20	1.43	34
Cell-F*	5°	60	9.2	9.2	100
Cell-F	23°	60	9.2	1.7	18.5

The CGW-AT samples on the other hand lost very little activity over the whole 68 days of storage and retained 80–90% of their activities.

The Cell-T samples retained only 60% of their original activity over the storage period. However, these samples were stored dry.

The M derivatives varied from a low of 13% activity retained to a high of 100% activity retained over a 42-day period; however, when wet the M(1:1) sample lost all activity within 30 days.

Considering the storage conditions employed and the storage times of each sample, the CGW-AT sample appears to be more stable than all other enzyme derivatives of trypsin tested. The M derivatives contained large quantities of enzymes

TABLE III

SUMMARY OF STABILITY STUDIES OF PAPAIN DERIVATIVES

Derivative	*Storage conditions*	*Original activity (mg/g)****	*Time in storage (days)*	*Final activity (mg/g)****	*Percent of original activity remaining*
CGW-AP1	5°	3.85	60	0	0
CGW-AP1	23°	3.85	60	0	0
CGW-AP8	5°	31.4	41	31.4	100
CGW-AP9*	5°	0.157	42	0.157	100
CGW-SP1	5°	1.49	60	0	0
CGW-SP1	23°	1.49	60	0	0
CGW-SP2	5°	1.84	60	0	0
CGW-SP2	23°	1.84	60	0	0
CGW-SP4*	5°	0.07	44	0.07	100
Cell-P*,†	5°	0.66	58	0.45	68
Cell-P*,††	23°	0.66	58	0.35	54
M-P*	5°	1.20	10**	1.20	100

* Crystalline papain, activity based on mg of crystalline papain coupled, assuming that the attached and free solution enzymes have identical activities. All other samples were coupled with a crude papain preparation having approx. 1/1000 of the activity per g of protein.

** Ran out of sample and discontinued assays.

*** See Table I for details.

† Represents cellulose derivative of crystalline papain.

†† Represents azo papain derivative of polyaminoacid.

but showed relatively low activities when assayed on casein substrate. The CGW derivatives, although they contain less activity per g, have higher percentages of active enzyme. The explanation is most likely that there is less diffusion control in the case of the CGW enzymes, while in the case of the copolymers, the protein substrate was unable to penetrate to the interior of the polymer.

Ficin

All the preparations maintained at 5° retained 100% of their original activity. The CGW-SF and the Cell-F when stored at 23° lost 66 and 81.5% of their activity, respectively. The results are presented in Table II. The CGW enzymes, particularly the CGW-AF, appear to be the more stable preparations.

Papain

The results shown in Table III indicate that the crystalline papain derivatives have greater stability than do the derivatives prepared with crude papain except for CGW-AP8. This derivative, however, has a much larger quantity of enzyme coupled per g and was prepared under slightly different conditions. Therefore, it should be considered separately from the other derivatives.

The CGW derivatives retaining activity appear to be more stable than the cellulose preparation. In the case of M derivative, not enough material was available and, therefore, no comparison could be obtained.

TABLE IV

SUMMARY OF STABILITY STUDIES OF GLUCOSE OXIDASE DERIVATIVES

Derivative	*Condition of storage*	*Original activity (mg/g)***	*Time in storage (days)*	*Final activity (mg/g)***	*Percent original activity*
CGW-ALGO	5°	6.0	68	6.0	100
	5°, in water	6.0	68	6.0	100
	23°	6.0	68	6.0	100
	23°, in water	6.0	68	6.0	100
CGW-HyGO	5°	10.7	68	10.7	100
	5°, in water	10.7	68	7.0	65
	23°	10.7	68	10.7	100
	23°, in water	10.7	68	10.7	100
CGW-SGO	5°	11.8	68	9.5	81
	5°, in water	11.8	68	8.6	73
	23°	11.8	68	8.6	73
	23°, in water	11.8	68	9.0	77
CGW-AGO	5°	10.1	68	10.1	100
	5°, in water	10.1	68	10.1	100
	23°	10.1	68	10.1	100
	23°, in water	10.1	68	10.1	100
Cell-GO*	5°	6.1	52	4.2	68.7
	5°, in water	6.1	68	1.0	16.4
	23°	6.1	68	3.5	57.3
	23°, in water	6.1	68	0.7	8.7
Soluble enzyme	5°	250 μg/ml	60	250 μg/ml	100
	23°	250 μg/ml	60	187 μg/ml	75

* Represents cellulose derivative of glucose oxidase.
** See Table I for details.

Glucose oxidase

The results, presented in Table IV, show the increased stability obtained by coupling glucose oxidase to an inorganic carrier. The increased stability appears to be as great with the alumina and hydroxyapatite carriers as with glass. The soluble enzyme maintained at 5° was more stable than Cell-GO.

CONCLUSIONS

Several general conclusions are apparent from the observed results. The inorganic derivatives are generally more stable than the enzymes covalently coupled to the organic polymers. The results also indicate that the azo derivatives are a great deal more stable than the sulfonamide derivatives during storage. In the case of the glucose oxidase the soluble enzyme had greater stability than the cellulose derivative.

The results also indicate that in the case of papain greater stability was achieved with crystalline enzyme covalently coupled to the inorganic carrier. The actual quantity of enzyme coupled per g of glass was equivalent to the cellulose derivatives but not the copolymer derivatives. Preparation M(4:1) (Table I) contained 800 mg of enzyme per g of derivative; however, when assayed on casein, the activity was only 41.0 mg/g of derivative. This represents approx. 5% of the total enzyme in the polymer.

The insolubilized enzymes covalently coupled to inorganic carriers such as porous glass appear to have superior stability during storage. They have increased thermal stability[10], excellent flow characteristics and enzyme activity is approximately equivalent to most insoluble enzyme derivatives covalently coupled to organic polymers.

In all studies the assumption was made that the activity of the insolubilized enzyme as determined from a standard curve prepared with soluble enzyme was valid.

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