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PEPSIN INSOLUBILIZED BY COVALENT ATTACHMENT TO GLASS: PREPARATION AND CHARACTERIZATION

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SUMMARY

1. Pepsin was covalently coupled to porous 96% silica glass particles.

2. The insoluble enzyme retained up to 65% of theoretical activity in a 5-min assay with hemoglobin substrate.

3. Almost total activity was observed during an assay at high substrate concentration over a long period of time.

4. The insoluble enzyme was found to retain activity for over 30 days during continuous operation.

INTRODUCTION

Enzymes have been covalently coupled to inorganic carriers[1–5]. These enzymes show increased storage and operational stability over enzymes coupled to organic derivatives[3–5]. However, no attempts have been made to covalently attach an enzyme requiring an acidic environment to inorganic carriers. Pepsin was chosen for this study because its pH optimum is around 2.0, and it rapidly denatures above pH 5.0[11]. Therefore, attachment of this enzyme must be carried out at acid pH. The enzyme was attached by activating its carboxyl groups with a water-soluble carbodiimide to effect amide bond formation with aminofunctional glass particles. Two grades of pepsin were used in order to determine, for potential commercial application, whether a more expensive highly purified enzyme or a less expensive crude enzyme could be used. It was of interest to learn whether any differences other than specific activity occurred when a crude enzyme and a purified enzyme were coupled to porous glass.

MATERIALS AND METHODS

Pepsin in two grades of purity, crude (grade 1:15 000) and 1 × crystallized

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(grade 1:65 000), was supplied by Wilson Laboratories, Chicago, Ill. The glass was obtained from Corning Glass Works, Corning, N.Y. Bovine hemoglobin was from Nutritional Biochemicals, Cleveland, Ohio. All other chemicals were reagent grade.

Preparation of pepsin coupled to glass

Porous 96% silica glass particles, 550 Å average pore diameter, 40/60 mesh particle size, were silanized with γ-aminopropyltriethoxysilane as previously described[1-3]. The alkylaminosilane–glass derivative was washed with acetone and air dried.

The pepsin was coupled to the aminofunctional glass as follows. 1 g of glass was suspended in 10 ml water containing 50 mg pepsin. 40 mg of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide, metho-*p*-toluenesulfonate were added and the pH was adjusted to and maintained at 4.0 with HCl for 30 min at room temperature. The reaction was continued at 6° overnight, after which the solid was harvested by filtration, washed exhaustively with 0.01 M HCl, and stored as a moist filter cake at 6°.

Enzyme assays

The assay procedure was essentially that described by HERRIOTT[6] using hemoglobin as substrate. A known weight of insolubilized pepsin suspended in 0.5 ml of 0.06 M HCl. The hydrolysis was carried out at 25° for the indicated times at a final pH of 2.0. The reaction was terminated by addition of 5 ml 5% trichloroacetic acid. The tubes were filtered and the trichloroacetic acid-soluble aromatic peptides were read at 280 nm against a substrate blank treated as described, but containing no enzyme.

Initial proteolytic activity

This is a kinetic term in which the assay was performed under conditions such that the rate of hydrolysis is linear with both time and enzyme concentration. The specific activity is expressed in $A_{280\ nm}$ units in the trichloroacetic acid filtrate per min per mg total protein coupled to the glass. The samples were assayed for 5 min, with increasing quantities of enzyme.

Protein hydrolyzing capacity

The assay was performed using a large excess of time and pepsin, *i.e.* under conditions expected to effect maximal hydrolysis. In this case the assays were carried out with 1 mg of native pepsin and compared with a quantity of glass containing 1 mg of pepsin for a period of 2 h.

pH profile

The pH–rate profile was performed using 2% hemoglobin which had been dissolved in 0.06 M HCl and then adjusted to the assay pH with HCl or NaOH. The assays were conducted as above except that the derivatives were suspended in 0.5 ml water rather than 0.06 M HCl.

Moisture content

Samples were dried to a constant weight in an oven at 105°. These values were employed for all determinations which were corrected for the moisture content of the derivative.

Protein determinations

The quantity of pepsin coupled to the glass was determined from the total arginine content.[7,8] The arginine content of native crystalline pepsin was taken at two residues per mole pepsin (molecular weight = 35 000)[9], whereas the arginine content of the crude material was determined directly as described below.

Arginine was liberated from the derivative by a 24-h hydrolysis under 6 M HCl[8]. The amount of arginine released was determined by the modified Sakaguchi reaction of MESSINEO[7], which in turn was modified as follows: (1) NaOCl was eliminated from Reagent B[7]. Its inclusion yielded a deep-red solution which failed to give the test with arginine. (2) Prior to color development primary amino groups were acylated with acetic anhydride, since they interfered with the test[12]. The colored product was determined at 520 nm. A molar extinction coefficient of $2.9 \cdot 10^4$ was obtained with arginine.

Operational and storage stabilities

Samples of insolubilized pepsin were stored at 6° in solutions of $(NH_4)_2SO_4$ and Na_2SO_4 at various concentrations and pH values. Attempts were made to dry the derivatives with acetone and methanol. The derivatives were stored for 30 days in the salt solutions and their activities compared to the original activity and the activity of samples stored as a wet cake at 6° for the same time period.

Operational stability was determined as follows: derivative columns containing 11.3 mg crystalline pepsin and 3.5 mg crude pepsin activity were prepared. The columns were operated at room temperature using 2% hemoglobin as substrate at a flow rate to give between 35 and 45% hydrolysis of the product. Losses in activity were determined by monitoring changes in percent hemoglobin hydrolyzed. The columns were operated continuously for more than 30 days in each case.

Thermal profile

Samples of both native and insolubilized pepsin were assayed at increasing temperatures and the Q_{10} values were determined for temperatures between 25 and 75°.

RESULTS

The quantities of protein coupled to two specific preparations of the alkylamine glass are given in Table I. These preparations were used for all the studies herein reported. Both crude and crystalline derivatives contained about 2% (w/w) protein. Several preparations were made with both grades of pepsin, and the reproducibility of the coupling method was determined by a statistical approach at the 95% confidence level. The results given in Table II show a standard deviation of less than $\pm$ 3%.

The protein hydrolyzing capacity values for the crude and crystalline derivatives listed in Table I were determined from the time course curves presented in Fig. 1. These data indicate that a quantity of glass containing 1 mg crude pepsin was able to hydrolyze 84% as much hemoglobin as 1 mg of the native preparation. Similarly, the crystalline derivative was able to hydrolyze 94% as much hemoglobin as the native enzyme (Table I, Fig. 1).

TABLE I

ARGININE AND PROTEI N CONTENT OF INSOLUBILIZED PEPSIN

All values were reproducible to at least ± 10%.

Enzyme prep.	*Arginine content (nmoles/mg derivative)*	*Activity (mg/g derivative)*	*Pepsin coupled (% of total wt.)**	*Protein hydrolyzing capacity ($A_{280\ nm}$ units/h)*	*% Native*	*Initial proteolytic activity ($A_{280\ nm}$ units/mg per min)*	*% Native*
Crude native	287	—	—	5.93	100	0.98	100**
Crude insolubilized	6	6.95	2.1	4.97	84	0.32	33
Crystalline native	—	—	—	6.60	100	4.20	100***
Crystalline insolubilized	0.99	11.3	1.74	6.10	94	2.70	65

* Represents percent of total protein coupled to the glass.
** Based on arginine content of crude native pepsin.
*** Percent pepsin coupled assumes crystalline pepsin molecular weight of 35 000 containing 2 residues arginine per mole.

Table I also lists the initial velocities for both crystalline and crude derivatives as compared to their respective native counterparts. These data were derived from the rate *vs.* enzyme curves depicted in Fig. 2. These results show that the crude derivative possessed a specific activity equal to 33% that of the soluble preparation, while the crystalline derivative was 65% as active as native pepsin.

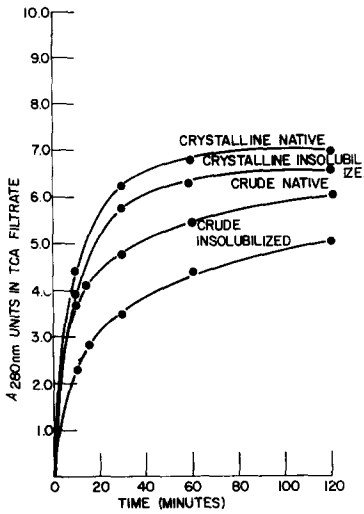
The pH profiles for both derivatives as compared with the native enzyme are given in Fig. 3. The ordinate is presented as the percent of the specific activity at pH 2.0, which was set at 100%. The only major difference was noted with the coupled crude enzyme, which was independent of pH between 1 and 3, but declined to 55% at pH 4.0. The profile of the crystalline adduct paralleled that of the native preparation.

TABLE II

REPRODUCIBILITY OF COUPLING PEPSIN TO POROUS GLASS

Each preparation was assayed 5 times. The statistical data were calculated by collectively using all the data. No differences were found between individual preparations. Activity is presented in μg as determined from a standard curve prepared with the appropriate quality enzyme preparation. The standard was also statistically treated, and the above data take this error into account. The relative activity of the crystalline enzyme was 5 times that of the crude per mg enzyme. Therefore although the activity in μg coupled is higher, the total activity is less.

Enzyme prep.	*Number of preparations*	*Sample wt. (mg)*	*Activity*	*95% limits*		*S.D. (μg activity)*
				Lower	*Upper*	
Crystalline	4	4.3	20.29	18.91	21.68	0.64
	4	3.9	19.21	17.71	20.71	0.70
Crude	2	3.7	68.05	66.39	69.71	0.68
	2	3.8	66.55	64.87	68.24	0.69



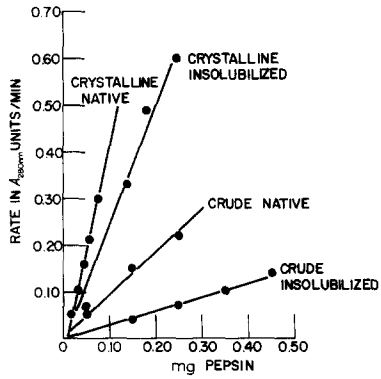


Fig. 1. Protein hydrolyzing capacity. Assays were performed using 1 mg pepsin and that amount of derivative containing 1 mg pepsin activity. TCA = trichloroacetic acid.

Fig. 2. Initial proteolytic activity. All assays were performed for 5 min using increasing quantities of native pepsin or the amount of derivative yielding the quantity of pepsin protein indicated on the abscissa.

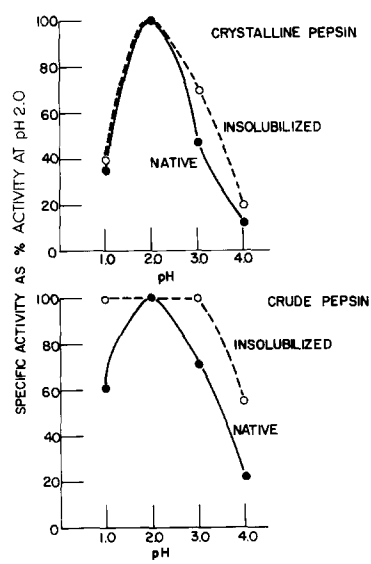


Fig. 3. pH profiles for crude and crystalline pepsin and their insolubilized derivatives. The specific activities were plotted as the percentage of the specific activity at pH 2.0, which was set at 100%.

TABLE III

STORAGE STABILITY OF INSOLUBLE PEPSIN

All samples were stored 30 days at 6°

Sample	*Storage conditions*	*Activity (mg/g derivative)*		*% Recovery*
		Original	*Final*	
Crude insolubilized	Satd. $(NH_4)_2SO_4$*	6.9	0.53	7.7
		5.6	0	0
		3.4	0	0
	Acetone dried	6.9	0	0
	17% Na_2SO_4*	6.9	0	0
		5.6	0	0
		3.4	0	0
Crude insolubilized	Abs. methanol	5.6	0	0
	Wet cake	5.6	5.6	100
Crystalline insol.	Satd. $(NH_4)_2SO_4$*	9.0	0	0
	17% Na_2SO_4*	9.0	0	0
	Wet cake	9.0	9.0	100

* Adjusted to pH 2.0 with HCl.

The insolubilized pepsin derivatives were stored for 30 days under various conditions. The results of these studies are given in Table III. It can be seen that the most satisfactory condition for storage was as a moist cake at 6°.

Operational stability was determined for both the crude and crystalline insoluble enzyme derivative. A total of 10 l of 2% hemoglobin was passed through the column containing crystalline enzyme in 25 days. Similarly, 15 l passed through the column containing crude enzyme in 40 days. At the time of the manuscript's preparation, both columns have not shown any losses in activity. They are still in operation and have hydrolyzed 90 g hemoglobin for the crystalline and 135 g hemoglobin for the crude derivative.

Thermal stability was determined by assaying at increasing temperatures. The results (Fig. 4) indicate that the native crude pepsin had greater thermal stability

TABLE IV

Q_{10} VALUES FOR NATIVE AND INSOLUBILIZED CRUDE PEPSIN

Q_{10} values represent ratio of the velocity at temperature $(T + 10)°$ to that at $T°$.

Sample	*Temperature*	Q_{10}
Native crude	35–45°	1.43
	45–55°	1.65
	55–65°	1.13
	65–75°	0.13
Crude insolubilized	35–45°	1.52
	45–55°	1.52
	55–65°	0.72
	65–75°	0.38

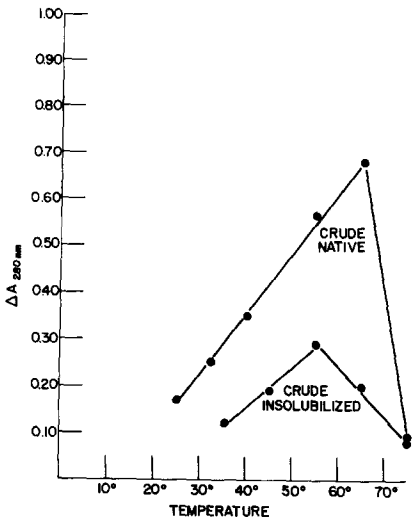


Fig. 4. Thermal profile of crude native and crude insolubilized pepsin. Assays were carried out at the indicated temperatures and the temperatures were plotted *vs.* the observed change in the absorbance in the trichloroacetic acid filtrate at 280 nm.

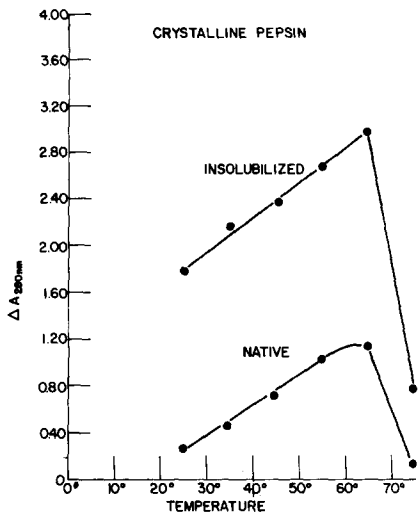


Fig. 5. Thermal profile of crystalline native and insolubilized pepsin. Assays were carried out at the indicated temperatures and the temperatures were plotted *vs.* the observed change in the absorbance in the trichloroacetic acid filtrate at 280 nm.

than the insolubilized derivative. The maximum Q^{10} value of the native derivative shown in Table IV (1.65 in the interval 45–55°) was greater than that for the insoluble enzyme (1.52 over the same temperature interval). The results for the crystalline derivative depicted in Fig. 5 and summarized in Table V show that the native enzyme is much more stable than the insolubilized one.

DISCUSSION

The total protein coupled to the aminofunctional glass was near maximal,

TABLE V

Q_{10} VALUES FOR NATIVE AND INSOLUBILIZED CRYSTALLINE PEPSIN

Q_{10} values represent ratio of the velocity at temperature $(T + 10)°$ to that at $T°$.

Sample	*Temperature*	Q_{10}
Native crystalline	25–35°	1.21
	35–45°	1.57
	45–55°	1.44
	55–65°	0.95
	65–75°	0.093
Insolubilized crystalline	25–35°	1.27
	35–45°	1.10
	45–55°	1.10
	55–65°	1.10
	65–75°	0.25

based on the available surface area (40 m^2/g glass) provided by the glass, assuming that the enzyme is perfectly rectangular and forms a monolayer on the glass surface.

The weight percentage of protein was approximately the same (2%, w/w, Table I) for both the crude and crystalline derivatives. However, the initial proteolytic activity retention was 65% native for the crystalline derivative as opposed to only 33% native for the crude adduct. Since the native crystalline enzyme is about five times as active as the native crude enzyme (Table I), the crystalline adduct is, therefore, 8 times as active as the crude derivative. The difference in relative initial activities is probably due to the fact that the protein content of the crude derivative was estimated from the arginine content of the starting material. This preparation contains only 20% pepsin at best (Table I—initial proteolytic activity for native crude and crystalline enzymes), but it was assumed that the composition of the attached protein in the crude derivative was identical with that of the starting material, *i.e.* that no component of the crude protein mixture was preferentially coupled.

This assumption is most likely erroneous. One cannot *a priori* assume that each component of the crude derivative coupled equally well to the glass. Attempts to assay the insoluble enzyme hydrolysate by other methods including total ninhydrin were unsuccessful because the glass–silane was also ninhydrin positive, the color changing greatly with slight changes in reaction conditions.

The differences observed in pH profile, initial velocity, thermal stability and specific activity strongly indicate that other components besides enzyme coupled to the glass. This is an important problem in studying insolubilized enzymes whether coupled to glass or other derivatives and we are presently exploring this problem in great detail.

The retention of enzymatic activity as measured by both the hydrolyzing capacity and initial velocity was good, when compared to the values observed for organic derivatives[4]. For example, trypsin insolubilized on a 1:1 copolymer of ethylene and maleic anhydride was found to retain only 5% of the native activity on casein substrate, although the retention of activity was considerably better against the synthetic ester substrate[4].

The hydrolyzing capacity may be a better criterion in assessing the relative activity of an insoluble derivative than is the initial velocity when using macromolecular substrates, such as casein or hemoglobin. The permeability of the substrate to the support can become rate limiting along with the formation of the enzyme–substrate complex. Maximum activity is achieved only when K_m is much smaller than substrate concentration. Permeability of the substrate to the support is, in turn, controlled by the support pore size and the relative charges of the support and substrate. The excess time and high substrate concentration employed in assessing the hydrolyzing capacity should minimize the diffusion-controlled permeation of the support by the substrate. The protein hydrolyzing capacity values for the insolubilized enzymes strongly indicate that most, if not all, the coupled enzyme is active. The crystalline derivative under the previously described assay conditions gave 94% of maximum activity when assayed in a large substrate excess and over a long period of time. However when assayed only five minutes the activity was 65% of theoretical. The difference is due to minimized diffusion control of substrate permeation into the carrier. One cannot avoid the effects of diffusion even with low mole-

cular weight substrates such as acetyl phenylalanylphenylalanine. Although the diffusion rate would be greater, if it is not much greater than the turnover rate of the enzyme it will still be limiting. Thus one would of necessity be required to use a large excess of the substrate to insure maximum activity.

For the purpose of these studies, we were only interested in large molecular weight substrates, these being of potential commercial importance.

The pH profile of the crystalline derivative paralleled that of the native enzyme (Fig. 3). As noted previously, the crude derivative was independent of pH between 1 and 3. One reason for the phenomenon would be the charge at the sufrace of the carrier. KATCHALSKI[10] has shown that apparent pH shifts can be explained by the fact that the pH at the surface of the carrier can be different from the observed pH in the surrounding medium. The crude pepsin contains not only enzyme, but other proteins, peptides, and low-molecular-weight materials capable of coupling to the glass. The coupled materials may place the attached enzyme in a microenvironment very different from that of the coupled crystalline pepsin.

Storage stability tests indicate that high salt concentrations inactivate the enzyme over a 30-day period. Efforts to dry the derivatives with acetone or methanol and lyophilization also inactivated the attached enzymes. However, they were quite stable when stored as moist cakes at 6°.

The operational stability of both insolubilized pepsin derivatives is good. Neither derivatives lost activity while operating continuously for 4 weeks.

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