IMMOBILIZED PRONASE *

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SUMMARY. Pronase was successfully coupled to an arylamino derivative of porous glass. The immobilized enzyme retains considerable activity against large and small substrates: bovine serum albumin, 57%; benzoyl-L-arginine ethylester, 27%; L-leucine-p-nitroanilide, 67%. Studies of enzyme stability, pH behavior and temperature dependence are presented.

The general significance and applicability of immobilized enzymes have been very capably reviewed (1-3). It is our specific purpose to use a mixture of immobilized proteases to completely hydrolyze peptides and proteins prior to amino acid analysis. This approach would eliminate the undesirable side reactions associated with the conventional acid hydrolysis (4-6). The feature of enzyme insolubility would hopefully reduce the operational difficulties inherent in the use of soluble enzymes for hydrolysis of peptides and proteins.

Pronase** was selected because of its very broad specificity (7-9). Porous glass (10) was chosen as the support for the following reasons: a) it is resistant to microbial attack, b) it permits a wide latitude of chemical conditions, and c) it permits rapid column flow under high pressure.

This report includes a description of the preparation of the pronase-glass derivative and its characterization in terms of specificity, stability, pH dependence and temperature dependence.

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** protease mixture from Streptomyces griseus (E C 3.4.4 group and E C 3.4.1. group). Other abbreviations: BSA, Bovine Serum Albumin; BAEE, N-α-Benzoyl Arginine ethylester; LPNA, Leucine-p-nitroanilide.

MATERIALS AND METHODS

Pronase (Lot P-5130), BSA, BAEE, LPNA, and Tris were supplied by the Sigma Chemical Company. The arylamino derivative of porous glass (11) was the generous gift of Dr. H. H. Weetall. All other chemicals were reagent grade.

Coupling of enzyme to glass by means of azo linkage was carried out according to Campbell, et al., (12) at pH 8.0 in 0.05 M Tris which was 0.025 M in $CaCl_2$. The reaction was followed by analyzing aliquots withdrawn at given time intervals. Total protein (13), BAEE activity and LPNA activity were determined.

Hydrolysis of BAEE was followed by the method of Schwert and Takenaka (14) with the Cary 15 and also by titration with a Sargent pH- stat (S-30240). LPNA hydrolysis was followed spectrophotometrically by monitoring p-nitroani line appearance at 405 nm. Proteolytic activity was followed by measuring the optical density (280 nm) of TCA soluble peptides.

RESULTS

The course of the enzyme coupling is shown in Figure 1. The resulting pronase-glass derivative contained 0.02 mg enzyme/1 mg glass. The immobilized pronase is active against both large and small substrates (Table 1). The enzyme may be reused many times without loss of activity as long as it is kept moist. It is stable stored in the moist form for at least 3 months.

The pH optima of the soluble and immobilized enzymes are similar, but the profile of the latter is considerably broadened at the optimum (Figure 2).

Michaelis-Menten kinetics apply for the hydrolysis of BAEE and LPNA by both the soluble and insoluble forms of the enzyme; K_m and V_m values appear

Table 1

Enzyme	Activity versus				
	BAEE		LPNA		BSA
	$V_m \left(\frac{M}{min}\right)$	K_m (M)	$V_m \left(\frac{M}{min}\right)$	K_m (M)	v(ΔOD/min/mg)
Soluble Pronase	1.94×10^{-5}	1.60×10^{-5}	1.00×10^{-4}	0.8×10^{-3}	0.20
Insoluble Pronase	0.52×10^{-5}	5.53×10^{-5}	0.67×10^{-4}	2.0×10^{-3}	0.12
$\frac{\text{Parameter for Insoluble Pronase}}{\text{Parameter for Soluble Pronase}}$	0.27	0.29	0.67	0.40	0.57

Table I. Activities of soluble and insoluble pronase. Buffer - 0.05 Tris, pH 8.0, 0.025 M in $CaCl_2$. Temperature = 25°C for LPNA and BAEE hydrolysis, 40°C for BSA hydrolysis.

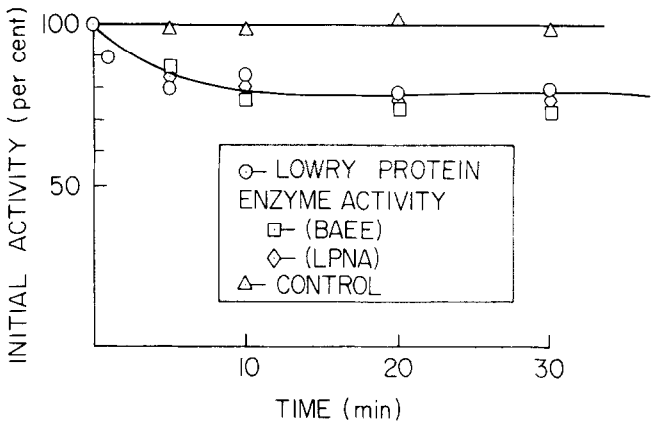

Figure 1. Depletion of enzymatic activities and total protein. One gram of diazotized arylamino glass was reacted with 100 ml of a solution of pronase-(1 mg/ml in 0.05 M Tris, pH 8.0, 0.025 M in $CaCl_2$). The temperature was 0°C.

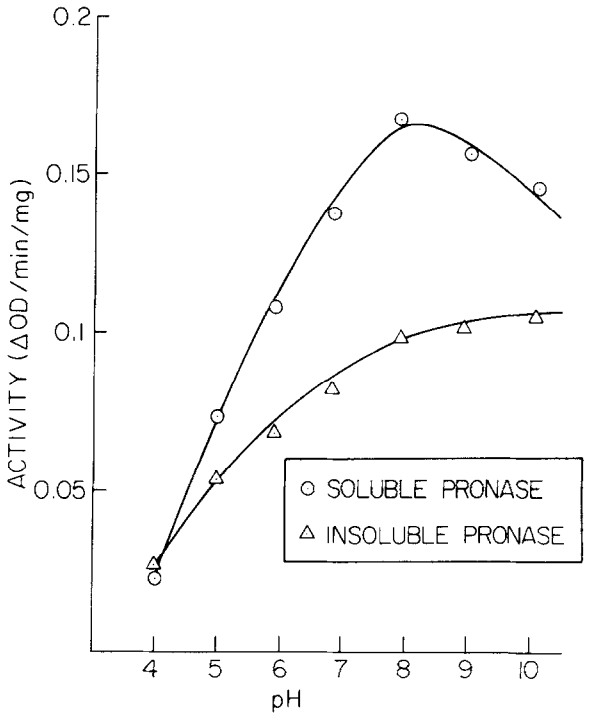

Figure 2. pH profiles of the soluble and insoluble pronase activities with the substrate BSA.

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in Table 1. The V_m values are given for identical enzyme concentrations on a mg/ml basis. The concentration of the insoluble enzyme is determined by de-

pletion and is therefore a reflection of total enzyme bound, not necessarily active enzyme.

The temperature dependences of the hydrolysis of LPNA by the soluble and insoluble enzymes are very similar. The activation energies calculated from Figure 3 are 11.5 kcal/mole and 13.0 kcal/mole for the soluble and insoluble enzymes respectively.

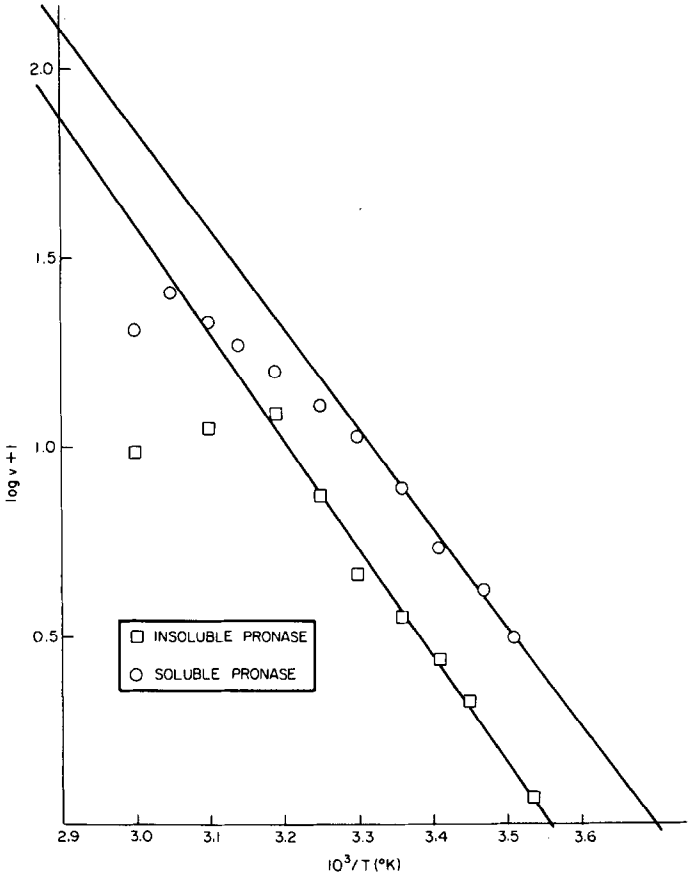

Figure 3. Arrhenius plots for soluble and insoluble pronase with LPNA as substrate. The values of ΔE are 11.5 kcal/mole and 13.0 kcal/mole respectively. The velocities are at enzyme saturation.

DISCUSSION

Cresswell and Sanderson (15) have coupled pronase to a co-polymer of leucine and p-aminophenylalanine. When this carrier is used, pronounced sub-

strate exclusion effects are exhibited. The pronase derivative of Cresswell and Sanderson retains about 20% of the native specific activity against BSA. Our preparation possesses 57% of the original activity against BSA which suggests that the enzyme is more accessible to substrate when porous glass is used as a support. A pepsin-glass derivative shows similar retention of activity against a substrate of high molecular weight (16).

The rate and extent of coupling of the BAEE-and LPNA-hydrolyzing components of pronase are identical (Figure 1). For these different proteins to contain an equal number of tyrosine residues of identical accessibility would be unlikely. An alternate explanation would require that pronase exists as an aggregate of enzyme components only one of which is covalently bonded to the insoluble carrier. Variation in $K_{m(sol)}/K_{m(insol)}$ (Table 1) from one enzyme component to another would be expected in this case.

Changes in V_m values on coupling of an enzyme could be explained in a number of ways. The new enzyme environment might well alter the structure of all molecules to an equal extent. Alternatively, some molecules of enzyme might be inactivated completely while the remainder would retain full activity. The similar pH optima for protein hydrolysis and the very small differences in activation energy of the soluble and insoluble LPNA-enzyme favor the second explanation.

Studies are now underway to determine the extent of peptide hydrolysis by a mixture of immobilized pronase and immobilized leucinea mino-peptidase.

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