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Immobilized Leucine Aminopeptidase Applications in Protein Chemistry

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ABSTRACT

Leucine aminopeptidase (EC 3.4.1.1) has been covalently bound to porous glass through an azo linkage. For the hydrolysis of leucine p-nitroanilide at pH 7.3 and 25°, $K_{m(app)}$ for the immobilized enzyme is higher than that of the soluble enzyme: 1.30 ● 0.2 and 0.53 ± 0.03 mM, respectively. However, at saturating levels of substrate the immobilized derivative and free enzyme have similar activities; k_{cat} values for the bound and free enzymes are 46 ± 5 and 46 ± 2 sec^{-1}, respectively. In addition, the pH and temperature dependences of the two enzyme forms are quite similar. These data suggest that the environment and conformation of the enzyme are not significantly changed after coupling. The apparent decrease in the substrate binding ability could be explained by a decrease in the effective diffusion coefficient of the substrate.

The insoluble enzyme is also active against peptide substrates. After treatment to remove contaminating proteases, immobilized leucine aminopeptidase was used successfully in

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sequencing experiments. The bound enzyme should be useful in total hydrolysis of peptides and proteins. The aminoethylated derivatives of the A- and B-chains of insulin were hydrolyzed essentially to completion. β-Lactoglobulin was hydrolyzed to the extent of 93% with immobilized leucine aminopeptidase and immobilized pronase.

Possible applications for immobilized enzymes exist in a number of areas including therapeutics, synthesis, analysis, and food processing [1-4]. It is also apparent that immobilized enzymes may serve as research tools. One area for such use would be protein structure studies. Specifically, we hope to prepare and employ a series of immobilized proteolytic enzymes for total hydrolysis, sequence determination, and proof of optical purity. Enzyme immobilization would permit the use of mixed proteases in high concentration for total hydrolysis. In all the applications the convenient separation of enzyme from digest is an advantage. Also, the insoluble enzymes are stable, reusable, and may be used in high concentration without the risk of contaminating the digest.

Leucine aminopeptidase is extensively used for amino acid sequence determination [5-7] and has also been used in attempts to hydrolyze proteins and peptides to free amino acids [8-10]. Although the importance of NH_2-terminus sequencing by leucine aminopeptidase has been diminished by the development of automatic sequencing systems, an immobilized leucine aminopeptidase derivative could be quite useful in placement of amides, tryptophan determination, proof of optical purity, identification of acid-labile catalytic intermediates, and identification of "affinity-labeled" side chains.

EXPERIMENTAL

Materials

Leucine aminopeptidase was "type IV" of the Sigma Chemical Co. The arylamine derivative of porous glass [11] was obtained from the Corning Glass Works. Tris ("ultra pure") and leucine p-nitroanilide (LPNA) were obtained from Schwartz/Mann. Insulin (Lilly Lot No. T2842) was the generous gift of Dr. Bruce Frank. Aminoethylation was carried out as described by Cole [12]; the chains were separated by chromatography on IRC-50 (Bio Rad) according to Humble, Deron, and Neuman [13]. All other chemicals were reagent grade.

Methods

Enzyme Coupling

Three hundred milligrams of the arylamino glass were suspended in 50 ml of 1 N HCl at 0° with mechanical stirring. Drops of sodium nitrite (0.5 N) were added until an excess was indicated with starch-iodide paper. After 15 min the glass was collected on a filter and washed with 200 ml of 3% sulfamic acid and 400 ml of distilled water. The coupling was carried out by adding the glass to a test tube containing a solution of leucine aminopeptidase (3 mg enzyme in 2 ml of 0.1 M Tris, pH 7.3, which was 1 mM in $MnCl_2$). The tube, connected to a constant torque stirrer, was rotated in an ice-water bath. The stirrer was stopped periodically and aliquots of supernatant were removed for assay. When reaction was complete the glass-enzyme derivative was filtered and washed with 200 ml of buffer. The preparation is stored at 4° C in a moist cake.

Determination of Enzyme Bound

The depletion of enzyme activity in the supernatant is used to calculate the amount of enzyme bound to the glass. Consideration should be made of any activity in the washings which represents loosely bound enzyme. For this particular system all of the activity was accounted for either in the form of tightly bound enzyme or free enzyme.

The amount of protein bound was also determined by amino acid analysis [14]. The control and enzyme-glass derivative were hydrolyzed with 6 N HCl in an evacuated, sealed tube at 110°C for 30 hr.

Enzyme Assays

The hydrolysis of LPNA was followed by monitoring the appearance of p-nitroaniline at 405 nm with a Cary-15 fitted with a thermostatted cell holder. The extinction coefficient employed was 9.9×10^3 1/mole·cm. The temperature was controlled to ±0.2°. The insoluble enzyme was assayed by filtering the reaction mixture at given time intervals and measuring the optical density of the filtrate. The samples were stirred at uniform rates in a water bath with a "Tri-R" MS-7 immersible stirrer.

The experimental errors in the rate determinations were within ±5%. The kinetic constants, k_{cat} and $K_{m(app)}$, were calculated by the method of Wilkinson [15]. These constants occur in the Michaelis-Menten equation

$$\frac{dp}{dt} = \frac{k_{cat}E_oS_o}{K_m + S_o}$$

which applies to the scheme

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

E_0 and S_0 are the initial concentrations of enzyme and substrate.

Peptide Hydrolysis

Digestions were performed with 1 mM solutions of A-chain in 0.2 N N-ethylmorpholine acetate, pH 7.3. A screwcap tube (15 ml) with baffled sides was used as a reaction vessel. The tube containing 5 ml of protein solution and 150 mg insoluble enzyme was rotated in a constant temperature bath (35°) by a constant torque motor. The enzyme employed in sequencing experiments was treated with diisopropylphosphofluoridate (DFP) and iodoacetate by standard procedures [6]. Preparations were stored under Tris buffer containing 1 mM $MnCl_2$. Samples (0.2 ml) withdrawn at 20, 40, 60, 80 min, and 24 hr were lyophilized, dissolved in pH 2.2 citrate buffer, and subjected directly to amino acid analysis [14]. No interference from peptides was evident.

RESULTS AND DISCUSSION

Preparation and Characterization of Immobilized Leucine Aminopeptidase

The time course of coupling appears in Fig. 1. The amount of enzyme coupled was determined both by depletion of activity in the supernatant and by amino acid analysis: 1.1 and 1.3% w/w, respectively. The average of these two values was used in calculating k_{cat}. We have used these enzyme preparations over many months without apparent loss of activity.

Both soluble and insoluble forms of the enzyme follow Michaelis-Menten kinetics (Fig. 2). For the immobilized and free forms of the enzyme, $K_{m(app)}$ values are 1.30 ± 0.2 and 0.53 ± 0.03 mM, respectively; k_{cat} values are 46 ± 5 and 46 ± 2 sec^{-1}, respectively. These data point up the importance of establishing the substrate dependence of the enzyme-catalyzed reaction for purposes of comparing the activities of soluble and insoluble enzyme forms. In this case, the Michaelis-Menten equation describes both reactions, but v_{ins}/v_{sol} (Fig. 3) varies as a function of substrate concentration until saturation is reached.

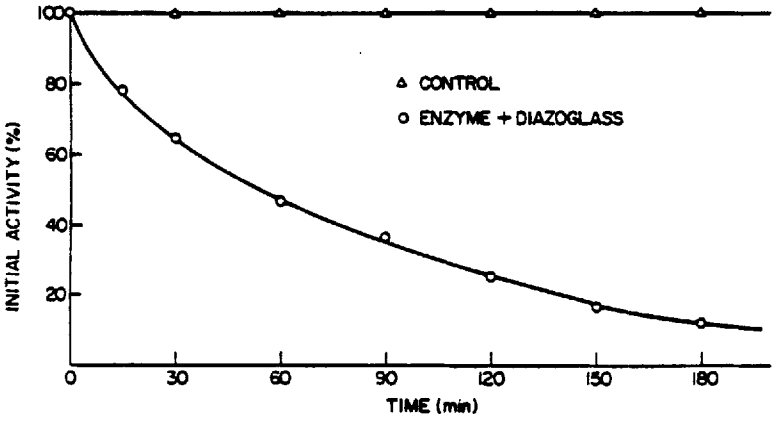

FIG. 1. Time course of enzyme coupling. Diazotized arylamino glass was reacted at 0° with 2 ml of leucine aminopeptidase solution (1.5 mg/ml in 0.1 M Tris-HCl buffer pH 7.3, 1 mM in $MnCl_2$). Activity was measured spectrophotometrically with leucine p-nitroanilide as substrate.

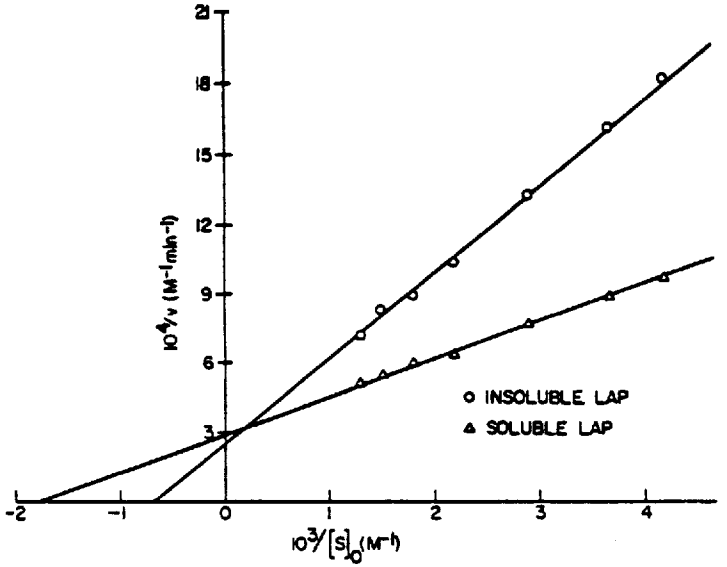

FIG. 2. Lineweaver-Burke plots for the soluble and insoluble forms of leucine aminopeptidase. The substrate is leucine p-nitroanilide at pH 7.3 and 25° C.

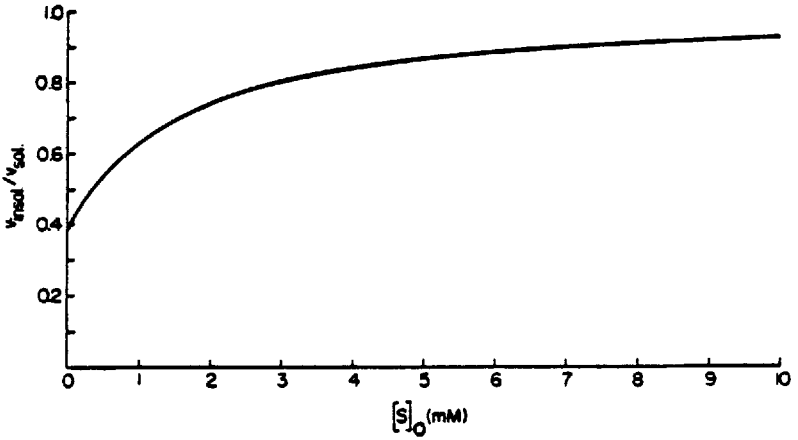

FIG. 3. Theoretical plot calculated from $v_{ins}/v_{sol} = [K_{m(sol)} + [S]_o]/[K_{m(ins)} + [S_o]]$. The respective K_m values employed were 0.53 and 1.30 mM for the soluble and insoluble enzymes.

This behavior reflects the differences in K_m for the two enzyme forms, i.e., when V_{max} is unchanged, Eq. (1) holds

$$v_{ins}/v_{sol} = \frac{K_{m,sol} + [S]_o}{K_{m,ins} + [S]_o} \qquad (1)$$

As shown in Fig. 3, v_{ins}/v_{sol} approaches unity at relatively large values of $[S]_o$.

A change in pH dependence often accompanies insolubilization of enzymes [16]. It is therefore important also to compare the activities of soluble and insoluble forms of an enzyme as a function of pH. Figure 4 shows similar $k_{cat}/K_{m(app)}$ profiles [17] for both enzyme forms. Arrhenius plots (Fig. 5) are also similar. For the free and immobilized forms the activation energies are 14.3 ± 1 and 12.5 ± 1 kcal/mole, respectively.

There are at least two explanations for complete retention of activity when an enzyme is immobilized. The most accessible side chains could be sufficiently far from the active site to allow covalent

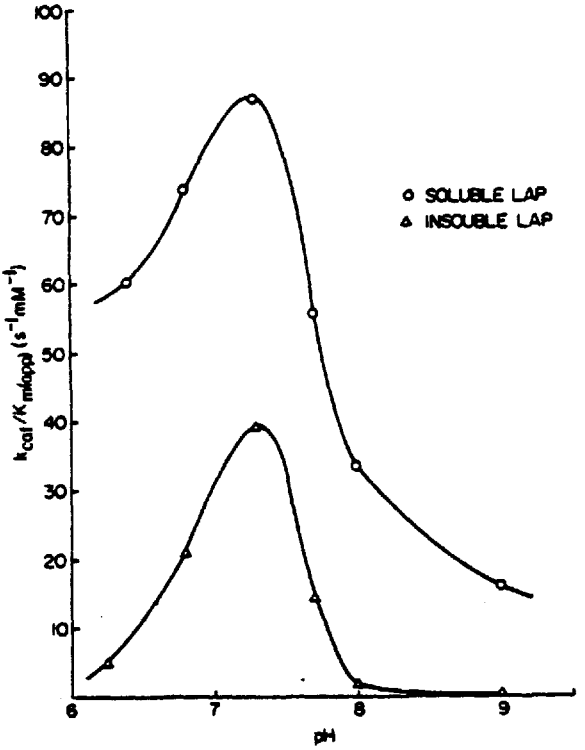

FIG. 4. pH profiles for hydrolysis of leucine p-nitroanilide catalyzed by soluble and insoluble leucine aminopeptidase. Tris buffer, 0.1 M and 1 mM in $MnCl_2$, was used for the entire pH range.

attachment without active site blocking. The second possibility would require heterogeneous binding areas on the insoluble support. Some enzyme molecules could be blocked on coupling while others could be activated.

Similarities in pH dependences and activation energies would favor the first situation. Also, Schwabe [18] found that a noncovalent complex of calcium phosphate gel and leucine aminopeptidase from dental pulp retained full activity. Although no comparisons of kinetic parameters for the soluble and insoluble forms of this enzyme were made, Schwabe suggested that the interaction of enzyme with support results in a "specific orientation" of the enzymes such that the active site is directed away from the support to face the solvent. Although our

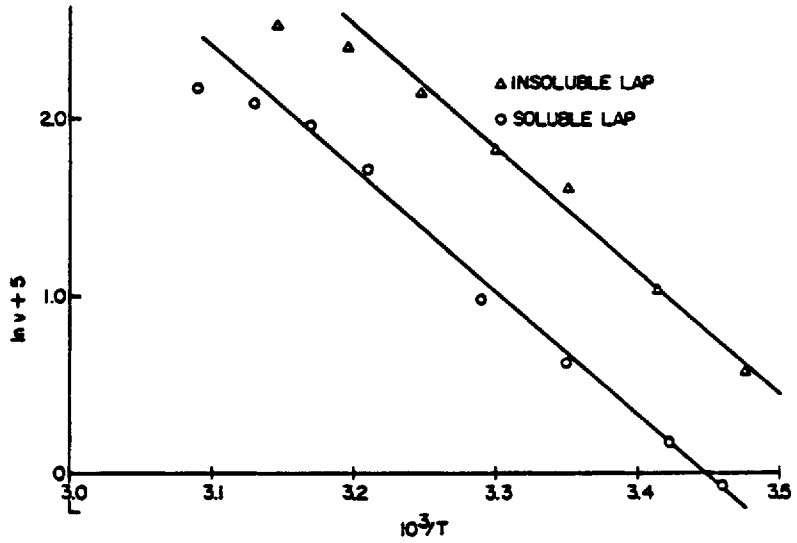

FIG. 5. Arrhenius plots for the hydrolysis of leucine p-nitroanilide by soluble and immobilized leucine aminopeptidase; enzyme concentrations were 1.2×10^{-8} and 7.5×10^{-8} M. Activation energies were 14.3 and 12.5 kcal/mole for the soluble and insoluble, respectively

system involves a different support and an enzyme from another source, we believe that a similar orientation of the enzyme prior to formation of a covalent complex could also explain the kinetic results presented here.

The apparent differences in substrate binding abilities of the two enzyme forms probably result from diffusion limitations [19, 20]. The effective diffusion coefficient of the substrate is changed by the presence of an "unstirred layer" on the support particle or by an interaction with the support itself.

Peptide Sequencing

The results of sequencing experiment (Fig. 6) indicate that the immobilized enzyme could be useful for NH_2-terminal degradation. The feature of enzyme reuse is valuable in that leucine aminopeptidase is a relatively expensive enzyme and treatment with DFP and iodoacetate is required only once. This enzyme derivative would be especially appropriate for methods involving examination of residual

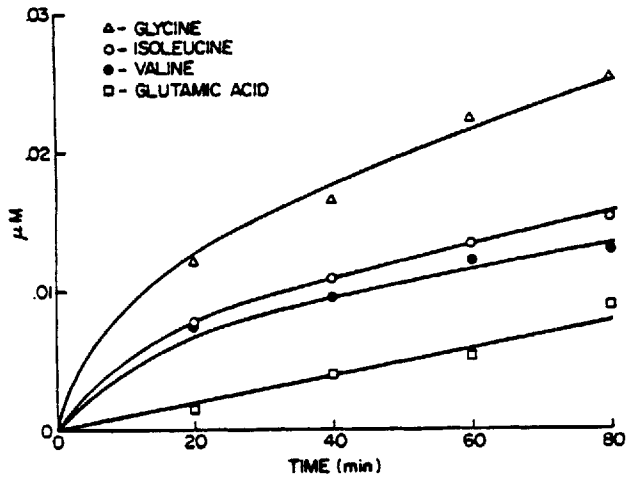

FIG. 6. Rate of release of amino acids from aminoethylated A-chain of insulin as catalyzed by immobilized leucine aminopeptidase. The expected sequence is gly-ileu-val-glu. See Experimental Section for details.

peptides, since reaction termination and product isolation is accomplished simply by filtration. Also, we hope to develop an automated system which would allow the release of a single amino acid residue per pass through the reactor.

Total Hydrolysis of Peptides and Proteins

The hydrolysis of aminoethylated A-chain of bovine insulin is essentially complete in 24 hr (Table 1). Aminoethyl (AE)-Cys appeared as a lone peak on the short column [14] at a position between the expected points of appearance of Lys and His; the color value for AE-Cys was taken to be 5% below that of Cys. Serine was not determined since Asn and Gln appear under this peak. The appearance of aspartic acid is the result of a desamido contaminant in this particular preparation of insulin (Asn 21 – Asp 21). The percent contamination found by our enzymatic method is in agreement with the manufacturers estimate based on electrophoretic studies.

The B-chain of insulin is also hydrolyzed nearly to completion (Table 2). This result was unexpected since X-Pro and Pro-X peptide bonds are hydrolyzed slowly or not at all by leucine aminopeptidase (LAP). The C-terminal tetrapeptide of the B-chain is Thr-Pro-Lys-Ala.

TABLE 1. Amino Acid Recovery from a 24-hr Digest of Aminoethylated A-Chain of Bovine Insulin by Immobilized Leucine Aminopeptidase

	Residues/mole A-chain		
	Found	Known	% Recovery
AE*-Cys	3.57	4.0	89
Ser	-	-	-
Glu	2.03	2	102
Gly	0.90	1	90
Ala	0.97	1	97
Val	1.80	2	90
Ile	0.90	1	90
Leu	1.84	2	92
Tyr	2.01	2	101
Asp	0.36	0.40	90
		Mean recovery	93

One would expect these residues to be recovered in low yield. Thr, Pro, and Lys are in fact recovered in low yield; 78, 60, and 80%, respectively. Alanine, however, appears to be completely released. This could be explained by a carboxypeptidase or trypsin-like contaminant in our LAP preparation.

For hydrolysis of β-lactoglobulin we chose to pretreat with immobilized pronase [21] which is mixture of proteolytic enzymes from S. griseus. Pronase digestion yields approximately 70% hydrolysis. On subsequent treatment with immobilized LAP increases, the recovery improves to 93% (Table 3). The recovery values for serine, glutamic acid, and aspartic acid were not obtained, since asparagine and glutamine elute under serine and were not determined. The low value of proline (25%) but high values elsewhere could be explained by the presence of carboxypeptidases in pronase. Also, di- or tri-peptide remnants containing proline could contain, in part, the amino acids not determined.

We feel that the data presented in this report indicate that complete protein digestion by mixtures of immobilized enzymes is certainly

TABLE 2. Free Amino Acids in a 24-hr Digest of Aminoethylated B-Chain of Insulin.

	Residues/mole B-chain		
	Found	Known	% Recovery
Lys	0.8	1	80
His	2.1	2	106
Arg	1.0	1	101
AE-Cys	1.8	2	88
Thr	0.8	1	78
Ser	-	-	-
Glu	1.9	2	97
Gly	3.0	3	101
Ala	1.9	2	95
Val	2.9	3	98
Leu	4.1	4	103
Tyr	2.0	2	100
Phe	3.7	3	103
Pro	0.6	1	60
		Mean recovery	93

feasible. Our present direction is to obtain immobilized enzymes which are specific for peptide bonds involving proline.

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TABLE 3. Amino Acid Recovery from a Digest of β-Lactoglobulin. The Protein Was Treated for 6 hr with Immobilized Pronase. This Digest Was Then Treated with Immobilized LAP Overnight

	Residues/mole β-lactoglobulin		
	Found	Known	% Recovery
Trp	2.0	2	100
Lys	15.1	15	101
His	1.6	2	80
Arg	3.1	3	103
Asp	12.4	-	-
Thr	7.5	8	94
Ser	-	-	-
Glu	14.7	-	-
Gly	3.3	3	110
Ala	12.9	14	92
Val	9.5	10	95
Met	4.1	4	103
Ile	9.4	10	94
Leu	21.1	22	96
Tyr	4.1	4	103
Phe	3.9	4	98
Pro	2.0	8	25
		Mean recovery	93

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