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# RANDOM PEPTIDE SYNTHESIS USING IMMOBILIZED ENZYMES IN HIGH CONCENTRATIONS OF ORGANIC SOLVENTS

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Trypsin, leucine aminopeptidase, and carboxypeptidase B were separately immobilized on controlled pore glass and reacted with a dipeptide substrate in high concentrations of either acetone or 1-propanol. Hydrolytic activity was demonstrated and evidence for the possible synthesis of peptide polymer is presented. Directed synthesis using amino acids and blocked amino acids as substrates was not successful.

## INTRODUCTION

Our previous report (1) showed that individual amino acids, when reacted in 76% ethanol at pH 7.0 with immobilized trypsin, do not bind to the carrier. However, recovery of dipeptide substrate and hydrolytic products under similar conditions typically resulted in product losses of 5–10% or more. Further investigation showed that the unaccounted-for hydrolysis products were in part associated with the carrier and only partly removed by washing the carrier with 2 M NaCl. Acid hydrolysis of the salt wash indicated that peptide polymers were present in the salt wash. This suggested the possibility that peptides were being synthesized and that reversal of enzymatic hydrolysis was taking place.

The endopeptidase trypsin and two exopeptidases were immobilized and studied. These studies are presented in this report. Our goal was to synthesize and identify, if possible, a dipeptide or peptide polymer.

## MATERIALS AND METHODS

### *Enzyme Substrates*

All substrates were CP or equivalent.

*Enzyme Immobilization*

*Immobilized Trypsin.* Trypsin was immobilized using the method previously described (1).

*Immobilized Leucine Aminopeptidase.* Five milligrams of leucine aminopeptidase (Worthington) was dialyzed against 1 liter of 0.005 M $MgCl_2$, pH 8.0, to remove $(NH_4)_2SO_4$. The dialysate was changed twice and the enzyme immobilized, using the coupling method described for trypsin (1).

*Immobilized Carboxypeptidase B.* Ten milligrams of carboxypeptidase B (Sigma) was coupled to 100 mg silanized 550 Å, 40/80 mesh controlled pore glass (CPG) as previously described (1).

*Activity Determination of Immobilized Enzymes*

*Immobilized Trypsin and Leucine Aminopeptidase.* The activity of immobilized trypsin was calculated from hydrolysis data using $10^{-3}$ M lysylglycine (Lys-Gly) as substrate in 90% 1-propanol, pH 7.0, 23°C.

*Immobilized Carboxypeptidase B.* The activity of immobilized carboxypeptidase B was determined using $\alpha$-glycyl-L-lysine (Gly-Lys) as substrate in 76% ethanol, pH 7.0, 23°C.

*Solvents and pH*

Solvents used in this study at various concentrations were acetone, ethanol, and 1-propanol. The apparent pH in all cases was 7.0, as measured on a Corning Model 12 pH meter equipped with L and N glass pH microelectrodes.

*Sample Preparation*

One or two milliliters of a $10^{-2}$ M aqueous substrate solution[1] was added to the solvent to produce the desired substrate and solvent concentrations. Immobilized enzyme derivative (100–200 mg) was then added and the pH adjusted to 7.0 with dilute NaOH. Also added was 0.01 M $CaCl_2$ and $MgCl_2$. Following incubation at 23°C in a stoppered flask with shaking, a 0.5 ml portion of the sample was removed, dried, and resuspended to volume with a pH 2.2 acetate buffer. A 20 $\mu$l sample was then injected into an amino acid analyzer for quantitation. If a mass balance was not observed, the soluble portion of each sample was acid hydrolyzed and reassayed on the amino acid analyzer. In addition, the IME portion was washed thoroughly

[1]Two individual amino acids or blocked amino acids or combinations of these.

with 2 liters of distilled water. Five milliliters of 2 M NaCl was added to each IME sample in a 25 ml flask, which was then stoppered and agitated for 24 h. Soluble portions of the IME NaCl wash were removed for amino-acid analysis and for acid hydrolysis followed by amino-acid analysis. IME samples were washed further with distilled water and acid hydrolyzed using 6 N HCl. The hydrolysates were then quantitated on an amino-acid analyzer. (The procedure is schematically represented in Fig. 1.)

*Nondirected Synthesis*

*Immobilized Trypsin (Trypsin IME).* One milliliter of $10^{-2}$ M Lys-Gly containing 0.01 M $CaCl_2$ was added individually to 9.0 ml acetone or 9.0 ml 1-propanol so that the final solvent concentration in both cases was 90%. Two hundred milligrams of immobilized trypsin was then added and the pH adjusted to 7.0 with dilute NaOH using a pH meter. The mixture was incubated a total of 7 days. During this time 0.5 ml samples were removed and assayed as described above.

*Immobilized Leucine Aminopeptidase (LAP IME).* One milliliter of an aqueous solution of $10^{-2}$ M Lys-Gly containing 0.01 M $MgCl_2$ was added to 4 ml 1-propanol so that the final solvent concentration was 80%. Two hundred milligrams of LAP IME was added and the mixture adjusted to pH 7.0 with dilute NaOH. The mixture was incubated and assayed as described above.

*Immobilized Carboxypeptidase B (CARB B IME).* One-half milliliter of $10^{-2}$ M $\alpha$-glycyl-L-lysine (Gly-Lys) was added to 4.5 ml 1-propanol so that the final solvent concentration was 90%. Two hundred milligrams of CARB B IME was added and the pH adjusted to 7.0. Incubation time, pH, temperature, and assay were as previously described.

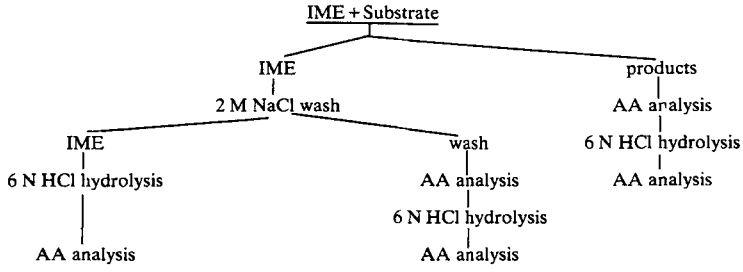


FIG. 1. Diagram of sample analysis.

TABLE 1. **List of Substrate Combinations Used in Directed Synthesis Experiments**

| |
|---|
| 1. L-lysine + glycine methyl ester |
| 2. L-arginine + glycine methyl ester |
| 3. Carbobenzoxy-L-arginine + glycine |
| 4. Carbobenzoxy-L-arginine + glycine methyl ester |
| 5. $\alpha$-*N*-benzoyl-L-arginine methyl ester + glycine |
| 6. Carbobenzoxy glycine + lysine methyl ester[a] |
| 7. L-arginine + L-aspartic |

[a]This substrate was tested with immobilized carboxypeptidase B; all others were with immobilized trypsin.

*Directed Synthesis*

Attempts to synthesize a dipeptide or peptide polymer were made by reacting CARB B IME or trypsin IME in 76% ethanol or 90% 1-propanol, pH 7.0, 23°C. Substrates were individual amino acids, blocked amino acids, or combinations of these (Table 1). After a suitable incubation time, a 0.5-ml sample was removed from the reaction flask and dried. Blocked amino acids were unblocked by addition of 0.5 ml concentrated HCl for 1 h at 37°C (2). The samples were again dried and resuspended to the original volume with pH 2.2 acetate buffer, then quantitated in an amino-acid analyzer.

## RESULTS

*Activity of Immobilized Enzymes*

The initial activities of the immobilized enzymes are listed in Table 2.

*Trypsin IME* (90% *Acetone*). Analysis of the soluble sample (Table 3) showed 10.3 nmol of Lys-Gly substrate was hydrolyzed. However, only 4.1 and 1.6 nmol of glycine and lysine, respectively, were recovered. Acid hydrolysis of the soluble samples yielded an additional 2.1 and 4.4 nmol of glycine and lysine, respectively. Finding additional dipeptides over and above the amount found in the soluble sample was significant, because it indicated the possibility that peptide polymers were being formed. Large molecular weight peptides would not be detected by the amino-acid analyzer. No amino acids or polymers were found in the soluble salt wash or acid hydrolyzed salt wash, indicating that amino acids or polymer (if present) were tightly bound to the carrier.

Acid hydrolysis of the washed IME indicated an additional recovery of 3.1 nmol for both glycine and lysine. Total recovery of hydrolyzed substrate was 90.2% for glycine and 88.3% for lysine.

TABLE 2. **Immobilized Enzyme Activities**

| Immobilized enzyme | Activity[a] (nmoles/min) |
|---|---|
| Trypsin | $2.7 \times 10^{-3}$ |
| Leucine aminopeptidase (LAP) | $8.0 \times 10^{-3}$ |
| Carboxypeptidase B | $1.3 \times 10^{-2}$ |

[a] Activity presented as nanomoles of product produced per minute under the conditions described in the test using 1-propanol with 200 mg of enzyme derivative. Activity was not determined in aqueous solutions. Protein content of each derivative was 14.0 mg/ml for trypsin, 7.5 mg/ml for LAP, and 1.0 mg/ml for carboxypeptidase.

*Trypsin IME* (90% 1-*Propanol*). Table 3 shows the results of an experiment in which the conversion of substrate to products went almost to completion. Of the 19.6 nmol hydrolyzed, 16.6 and 17.4 nmol of glycine and lysine, respectively, were recovered from the soluble samples. The same soluble samples after acid hydrolysis gave an additional 1.9 nmol of glycine and 1.8 nmol of lysine. The additional amino acids found in the acid hydrolyzed soluble samples indicated peptide polymer formation other than those of the substrate and products. No additional peaks were observed on the chromatogram from the amino-acid analyzer.

A 2 M NaCl wash of the sample IME yielded no additional amino acids or peptides. Acid hydrolysis of these samples yielded small amounts of glycine and lysine, which indicated the possibility of peptide polymer formation. Acid hydrolysis of the insoluble control and sample IME did not indicate additional amino acids were bound to the carrier. Total recovery of the missing amino acids was 96.9% for glycine and 100.0% for lysine.

*Leucine Aminopeptidase IME* (80% 1-*Propanol*). Table 3 shows that 6.6 nmol of Lys-Gly was hydrolyzed but only 1.1 nmol of both glycine and lysine was recovered from the soluble samples. The same samples when acid hydrolyzed yielded an additional 2.4 nmol of glycine and 2.1 nmol of lysine. The soluble and acid hydrolyzed salt washed samples gave no additional amino acids.

Acid hydrolysis of control and sample IMEs showed an additional recovery of 2.1 nmol of glycine and 0.8 nmol of lysine for the sample IME. Total recovery of hydrolyzed substrate was 84.8% for glycine and 60.6% for lysine. The low total recovery may represent losses due to the distilled water washing step between the soluble acid hydrolysis step and the salt washing procedure. This step was not quantitated due to the large volume of filtrate involved (2 liters).

TABLE 3. **Nondirected Synthesis Data**

| | Trypsin IME (90% acetone) | | Trypsin IME (90% 1-propanol) | | Leucine aminopeptidase IME (80% 1-propanol) | | Carboxypeptidase B (90% 1-propanol) | |
|---|---|---|---|---|---|---|---|---|
| | Total[a] | Recovered[a] | Total[a] | Recovered[a] | Total[a] | Recovered[a] | Total[a] | Recovered[a] |
| Soluble | | | | | | | | |
| Lys-Gly | 20.0 | — | 20.0 | — | 20.0 | — | 20.0 | — |
| Lys-Gly hydrolyzed | 10.3 | — | 19.6 | — | 6.0 | — | 7.7 | — |
| Gly recovered | — | 4.1 | — | 16.6 | — | 1.1 | — | 3.4 |
| Lys recovered | — | 1.6 | — | 17.4 | — | 1.1 | — | 3.3 |
| Soluble and acid hydrolyzed | | | | | | | | |
| Gly recovered | | 2.1 | | 1.9 | | 2.4 | | |
| Lys recovered | | 4.4 | | 1.8 | | 2.1 | | |
| 2 M NaCl wash | | | | | | | | |
| Soluble | | | | | | | | |
| Gly recovered | | | | | | | | |
| Lys recovered | | | | | | | | |
| Soluble and acid hydrolysis | | | | | | | | |
| Gly recovered | | | | 0.5 | | | | 0.1 |
| Lys recovered | | | | 0.4 | | | | 0.1 |
| Insoluble and acid hydrolysis | | | | | | | | |
| Gly recovered | | 3.1 | | | | 2.1 | | 0.3 |
| Lys recovered | | 3.1 | | | | 0.8 | | 0.5 |
| Total recovered Amino acids | | Gly 90.2% Lys 88.3% | | Gly 96.9% Lys 100.0% | | Gly 84.8% Lys 60.6% | | Gly 49.4% Lys 50.6% |

[a] In nanomoles.

TABLE 4. **Summary of Nondirected Synthesis**

| Immobilized enzyme | Substrate | Solvent | Hydrolysis | Comments |
|---|---|---|---|---|
| Trypsin | $10^{-3}$ M Lys-Gly | 90% Acetone | + | Possible peptide polymer formation in soluble sample.<br>No polymer in salt wash.<br>Some amino acids/polymer adsorbed to carrier.<br>Unidentified peak in control and experimental soluble sample. |
| Trypsin | $10^{-3}$ M Lys-Gly | 90% 1-Propanol | + | Possible peptide polymer formation in soluble sample and salt wash.<br>No amino acids/polymer adsorbed to carrier.<br>No additional unidentified peaks observed. |
| Leucine aminopeptidase | $10^{-3}$ M Lys-Gly | 80% 1-Propanol | + | Possible peptide polymer formation in soluble sample.<br>No polymer in salt wash.<br>Some amino acids/polymer adsorbed to carrier.<br>No additional unidentified peaks observed. |
| Carboxypeptidase B | $10^{-3}$ M Gly-Lys | 90% 1-Propanol | + | No polymer formation in soluble sample.<br>Small amount polymer found in salt wash.<br>Some amino acid/polymer adsorbed to carrier.<br>No additional unidentified peaks observed. |

TABLE 5. **Directed Synthesis**

| Blocked amino acid substrates[a] | | Blocked peptide product | | Unblocked product |
|---|---|---|---|---|
| CBZ-Arg+Gly ME | Trypsin, IME → | CBZ-Arg-Gly ME | Conc. HCl, 37°C, 1 h → | Arg-Gly |
| CBZ-Gly+Lys ME | Carboxypeptidase B, IME → | CBZ-Gly-Lys ME | Conc. HCl, 37°C, 1 h → | Gly-Lys |

[a] CBZ: Carbobenzoxy—blocked $NH_2$.
ME: Methyl ester—blocked COOH.

*Carboxypeptidase B* (90% 1-*Propanol*). Table 3 shows 7.7 nmol of substrate hydrolyzed and 3.4 and 3.3 nmol of glycine and lysine, respectively, recovered. No additional amino acids were found in the acid hydrolyzed soluble samples. A small amount of peptide polymer was found in the acid hydrolyzed salt wash, which indicated the possibility of polymer formation. Acid hydrolysis of control and experimental IMEs yielded an additional 0.3 nmol of glycine and 0.5 nmol of lysine for the sample IME. Total nanomoles recovered were 49.4 and 50.6% for glycine and lysine, respectively.

Since the distilled water washing of the IME following removal of the soluble sample was not quantitated because of the large volume of filtrate (2 liters), it was possible for loosely bound amino acids or polymer to be washed off and lost. This could explain low total recovery of this experiment. Table 4 gives a summary of the work performed on nondirected synthesis.

### *Directed Synthesis*

Table 5 shows two representative experiments from this study. In addition to amino-acid analysis, thin layer chromatography was also used to identify substrates and products. All results to date have been negative. No synthesized peptides have been observed.

## DISCUSSION

Our data on random synthesis have shown the possibility of peptide polymer formation based on additionally recovered amino acids in acid hydrolyzed samples compared with nonacid hydrolyzed samples. Since only low molecular weight molecules are detected by the amino-acid analyzer,

the peptide polymer formed must be of sufficiently high molecular weight to escape detection. However, we have not yet identified these polymers.

Recovery of additional unaccounted-for hydrolysis products from acid hydrolyzed salt washes and from acid hydrolyzed IME also indicated the presence of peptide polymer. One must assume that peptide polymer was in some way associated with the carrier, perhaps by nonspecific hydrogen bonding.

It will be noted that total recovery of substrate and products was observed only when the reaction went to completion (Table 3). Also, in this experiment no additional amino acids were found associated with the IME. This probably represents a final equilibrium condition between polymer formation and substrate hydrolysis.

Amino acids recovered in the other synthetic experiments (Table 3) were 90% or less of theoretical. In all cases some additional products were found associated with the same IME. Previous observations and the results presented in Table 3 indicate that recovery of Lys has typically been less than that of Gly. This difference was usually greater at solvent concentrations optimal for enzymatic activity. Lys and Gly reacted with trypsin at the optimum ethanol concentration were *not* adsorbed to the carrier, yet Lys could not be accounted for. It seems that polymer formation might be directed more toward producing Lys-Lys-peptide bonds in a manner similar to the mechanism described by Wally and Watson (3). They demonstrated transpeptidation by reacting a mixture of soluble trypsin and chymotrypsin with L-lysyl-L-tyrosyl-L-lysine and found that, in addition to lysine, tyrosine, lysyl-tyrosine, and tyrosyl-lysine, lysyl-lysine was also formed. They suggested that the hexapeptide Lys-Tyr-Lys-Lys-Tyr-Lys was formed as an intermediate that breaks down to Lys-Tyr, Lys-Lys, and Tyr-Lys.

Amino-acid ester formation could have been a factor that influenced our observations on directed synthesis. Glazer (4) found no evidence of esterification when trypsin and lysine were reacted with *low* concentrations of various alcohols. However, significant transesterification was found when lysine methyl ester was reacted with trypsin in low concentrations of various alcohols. Thin layer chromatography for the presence of ester in the product proved negative.

For those directed synthesis experiments, which used lysine and glycine methyl ester, some transesterification may have occurred that could have influenced the results.

Yamashita et al. (5) studied the "plastein"[2] productivity in some soluble proteases in aqueous systems and found that soluble trypsin in aqueous systems was not "plastein" productive at any pH. If the peptide polymers we

[2]A reaction that includes condensation and transpeptidation.

have indirectly observed do represent a plastein reaction, then immobilized trypsin, at least in high concentrations of organic solvents, must now be considered plastein productive.

Reversal of enzymatic hydrolysis for $\alpha$-chymotrypsin has been documented in low (6) and high (7) concentrations of ethanol. In the latter case, the enzyme was immobilized.

At present several preliminary experiments have been run in our laboratory with immobilized $\alpha$-chymotrypsin in 76% ethanol using dipeptides and blocked and unblocked amino acids as substrates. These experiments have not been successful. In fact, hydrolysis in 76% ethanol was not demonstrated, even though the immobilized enzyme was found to be active in an aqueous assay.

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