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SYNTHESIS OF ASPARTAME PRECURSOR BY SOLID THERMOLYSIN IN ORGANIC SOLVENT

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Summary

The enzymatic synthesis of a peptide compound was carried out successfully in homogeneous organic solvent.

Solid Thermolysin was found to catalyze the synthetic reaction of N-benzyloxycarbonyl-L-aspartyl-L-phenylalanine methyl ester (Z-APM; a precursor of sweetner Aspartame) from N-benzyloxycarbonyl-L-aspartic acid (Z-L-Asp) and L-phenylalanine methyl ester (L-PheOMe) in a 98 percent organic medium (ethylacetate:benzene:methanol:water=50:29:19:2). The dissolution of enzyme was not observed. The optimal pH shifted to acidic side by 1.0 pH unit, compared with that in aqueous medium. The enzymatic activity of solid thermolysin with an average size of 3.4×9.5 μm was determined to be 0.18 μmoles-product/(mg-solid)•h under the initial concentrations of L-PheOMe of 0.1M and Z-L-Asp of 0.05M, and at pH 6.0 and 40 °C.

INTRODUCTION

The synthesis of peptide compounds is complicated by the followings: chemical protections of functional groups other than the target amino- and carboxy-groups, and poor solubilities into aqueous media of amino acids and their derivatives and other peptide compounds. The former is solved by an enzymic method, because generally the enzyme is specific for the reaction between the target functional groups. The poor solubilities can be solved by using an organic solvent. Therefore, the synthesis of peptide compound could well be carried out enzymatically in organic media.

Oyama et al. (1981) succeeded in the synthesis of N-benzyloxycarbonyl)-L-aspartyl-L-phenylalanine methyl ester (Z-APM; the precursor of the synthetic sweetner Aspartame) from N-benzyloxycarbonyl-L-aspartic

acid (Z-L-Asp) and L-phenylalanine methyl ester (L-PheOMe) in an apparent single phase of the organic solvent by using immobilized Thermolysin. However, even in this procedure, the enzymatic reaction proceeded in aqueous medium filled in the inner side of the immobilization carrier; namely, it is a two-phase reaction.

In this work, the synthesis of Z-APM by thermolysin has been attempted in homogeneous organic solvent.

EXPERIMENTAL

Thermolysin powder with an average size of 3.4×9.5 μm and a purity of 69.1% was supplied from Daiwa Kasei Co. (Japan). The enzyme was used without further purification. All other chemicals used were of reagent grade. L-PheOMe and Z-L-Asp with working concentrations of 0.1 and 0.05M, respectively, were dissolved in a 98 percent organic medium (ethylacetate:benzene:methanol:water=50:29:19:2) at pH6.0. The batchwise reaction was started by the addition of a given amount of solid thermolysin to the substrate solution at 40 or 70℃. The reaction mixture was shaken at 132 oscillations per min. The concentration of product was determined by HPLC (column; Zorbax BP-ODS (Shimadzu Co., Japan), carrier; 0.05M(pH2.5) KH_2PO_4/CH_3CN (60/40 in volume) containing 0.1% pentane sulphonic acid, detection; absorption at 210 nm).

RESULTS AND DISCUSSION

The product was identified as a complex of Z-APM and L-PheOMe by NMR and IR spectra, and HPLC analysis. Figure 1 shows the pH-dependency of enzymatic activities of solute thermolysin in aqueous media and of solid thermolysin in organic media, where the unsymmetrical profile in solid thermolysin is attributed to the difference in the initial concentration of substrates as shown in the legend of Fig.1. The optimum pH shifted to the acidic side by 1.0 pH units.

Figure 2(a) shows the time course of the synthesis of Z-APM•L-PheOMe complex. Figure 2(b) represents the dependency of initial reaction rate on the amount of enzyme, indicating that the reaction rate is proportional to the amount of enzyme. From a slope of the straight line in Fig.2(b), the specific activity of solid thermolysin was estimated to be 0.18 μmoles-product/(mg-solid)•h at pH6.0 and 40℃. Assuming that the active enzyme molecules are localized in the external surface of the solid, the enzymatic activity per one molecule of active enzyme was estimated to be $(2.5\text{-}3.9)\times10^{-21}$ moles-product/molecule•h, whereas the value of a density of crystalline thermolysin 1.176 g/cm^3 (Colman P.M. et al, (1972)) was used and the radius of thermolysin molecule, as a sphere, was estimated from the empirical relationship between the surface area of protein molecule A, and its molecular weight MW, namely $A=5.6\cdot MW^{2/3}$ (Hamada K., 1979). The activity thus estimated was roughly equal to that obtained for solute enzyme (2.7×10^{-21} moles-product/molecule•h) in

aqueous medium at optimal pH 7.0 and 40℃. This result means that no much water is needed for the catalysis of thermolysin; no more than 2%.

Figure 3 shows that the time course of reaction at 70℃ and pH6.0. The reaction was strongly decelerated, comparing with the result at 40℃ shown in Fig.2, probably due to the thermal inactivation of enzyme or the lowering of the tolerance of enzyme for the organic solvent caused by the loosening of the protein molecule.

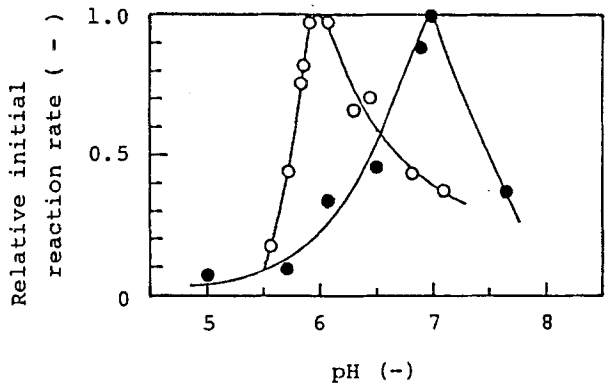

Fig.1 pH-dependency of enzymatic activities of solute thermolysin in aqueous media and of solid thermolysin in organic media at 40℃. Thermolysin; solute (●), solid (O). Reaction conditions: solute enzyme; [L-PheOMe]=0.02M, [Z-L-Asp]=0.02M, [$CaCl_2$]=0.02M, and [NaCl]=0.03M, solid enzyme; [L-PheOMe]=0.008M (pH>6.0) or 0.1M (pH<6.0), [Z-L-Asp]=0.004M (pH>6.0) or 0.05M (pH<6.0), [$CaCl_2$]=zero M, and [NaCl]=zero M.

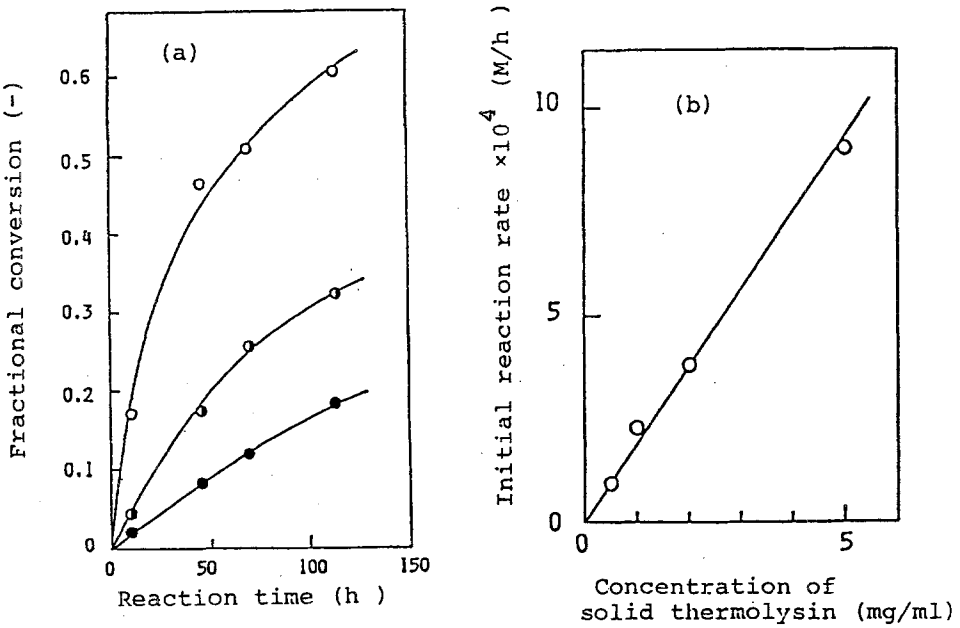

Fig.2 (a) Synthesis of Z-APM at 40℃ and pH6.0 by solid thermolysin. Concentration of enzyme (in mg/ml); 0.5 (●), 1.0 (◑), and 5.0 (O). (b) Dependency of the initial reaction rate on the concentration of enzyme.

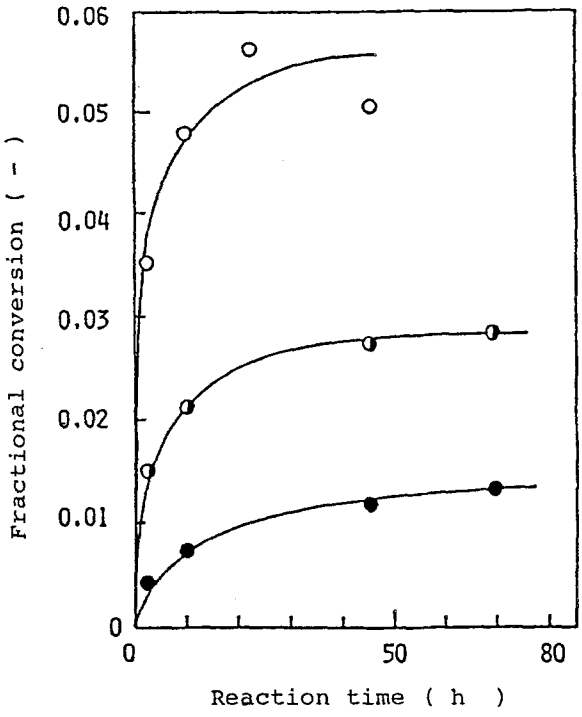

Fig.3 Synthesis of Z-APM at 70℃ and pH6.0 by solid thermolysin. Concentration of enzyme (in mg/ml); 0.5 (●), 1.0 (◑), and 5.0 (○).

References

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