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Note

Determination of esterase activity of papain by high-performance liquid chromatography

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High-performance liquid chromatography (HPLC) is used extensively to determine the concentrations of the reactants and products of chemical reaction. For example, specific tests for enzymes such as 5′-nucleotidase, guanase, xanthine oxidase and adenosine deaminase can be performed by HPLC[1–3] and the kinetic parameters of adenosine deaminase can also be measured[4].

Papain is a proteolytic enzyme from plants and possesses a stereospecific esterase activity on appropriate synthetic compounds. Several N-acyl-D,L-amino acid methyl esters and phenylhydrazines have been resolved by papain[5,6]. However, owing to the immiscibility of N-acyl-D,L-amino acid methyl esters in aqueous solution, the resolution reaction with papain was carried out in an aqueous–organic solvent mixture and the conventional pH-stat method was not suitable for enzyme assay in a non-aqueous solvent.

In this work, HPLC and pH-stat methods were compared for the determination of the kinetic parameters of papain for the water-soluble substrate, N-benzyl-L-arginine ethyl ester in the aqueous phase. Other water-immiscible substrates, N-acyl amino acid methyl esters, were also introduced and their kinetic parameters for the esterase activity of papain were determined by HPLC in the non-aqueous phase.

EXPERIMENTAL

Materials

Papain was purchased from E. Merck (G.F.R.), with a potency of 3.5 mAnson units/mg, and was used without further purification.

D,L-Methionine (Met), D,L-phenylalanine (Phe), benzyloxycarbonyl chloride (Cbz-Cl) and S-butyloxycarbonyl-4,6-dimethyl-2-mercaptopyrimidine were obtained from the Protein Research Foundation, Japan.

N-Benzoyl-L-arginine ethyl ester (BaEE) and 2-mercaptoethanol were purchased from Sigma (U.S.A.) and ethylenediaminetetraacetatic acid (EDTA) from Koch-Light (Great Britain).

The diazomethane in etheral solution was prepared according to the established method[7]. The N-benzyloxycarbonyl (Cbz-) amino acid was synthesized by the method of Fletcher and Jones[8] and further methylated with diazomethane[9]. The N-

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butyloxycarbonyl (Boc-) amino acid was synthesized by the established method[10] and methylated with diazomethane[9].

Apparatus

The pH-stat consisted of a Metrohm Herisau E512 pH meter, an Impulsomat E473 titrator and a Dosimat E412 autoburette.

The HPLC system included two Waters Model 6000 pumps, a Waters UK-6 valve-loop injector, a Waters Model 450 variable-wavelength UV detector and a Waters Model 600 solvent programmer. All of the columns were pre-packed by Waters Assoc.

Papain assay with BAEE as substrate

pH-stat method. The reaction was carried out at 37°C, with 10 ml of reaction mixture containing various concentrations of BAEE (0.005, 0.01, 0.015 and 0.02 *M*), 0.005 *M* mercaptoethanol, 0.002 *M* EDTA, 0.3 *M* sodium chloride and 0.5 mg of native papain. The reaction mixture was immediately adjusted to pH 6.2 and kept at this pH by addition of 0.1 *M* sodium hydroxide solution. The rate of hydrolysis was measured from the rate of consumption of alkali. One unit of enzyme activity was defined as the activity that hydrolysed 1 μmole/min of BAEE at 37°C[11].

HPLC method. The reaction conditions were as described in the pH-stat method. After reaction for 5 min, the reaction was terminated by the addition of 2 ml of 30% acetic acid. The reaction mixture was filtered through a 0.45-μm millipore filter and the filtrate was analysed by HPLC. The amount of the product was linearly related to the peak height and the rate of the enzyme reaction was accurately determined from the peak height of benzoylarginine.

Papain assay with N-acyl-D,L-amino acid methyl ester as substrate by HPLC method

The papain assay was carried out with a reaction mixture of 8 ml of methanol–0.05 *M* phosphate buffer, pH 6.5 (40:60) containing various concentrations of N-acyl-D,L-amino acid methyl ester, incubated at 37°C for 5 min with stirring. The reaction was terminated by addition of 2 ml of 30% acetic acid. The reaction mixtures were filtered through a 0.45-μm Millipore filter and the filtrates were analysed by HPLC. The rate of enzyme reaction was determined from the peak height of the N-acyl amino acid product.

RESULTS AND DISCUSSION

The amount of benzoyl-L-arginine produced from benzoyl-L-arginine ethyl ester by papain hydrolysis was measured by HPLC (Fig. 1). The reaction rate and kinetic parameters were determined by the HPLC and pH-stat methods, respectively, and compared with each other (Figs. 2 and 3). The kinetic parameters, the Michaelis constant, K_m and the maximal initial velocity, and V_{max}, determined by the pH-stat method were slightly higher than those obtained by the HPLC method, because the reaction rate measured by the pH-stat method was the initial rate rather than the average rate in 5 min as in the HPLC method.

The kinetic parameters of papain esterase activity for the substrates, N-Cbz-D,L-Phe-OCH_3, N-Boc-D,L-Phe-OCH_3 and N-Cbz-D,L-Met-OCH_3, were also deter-

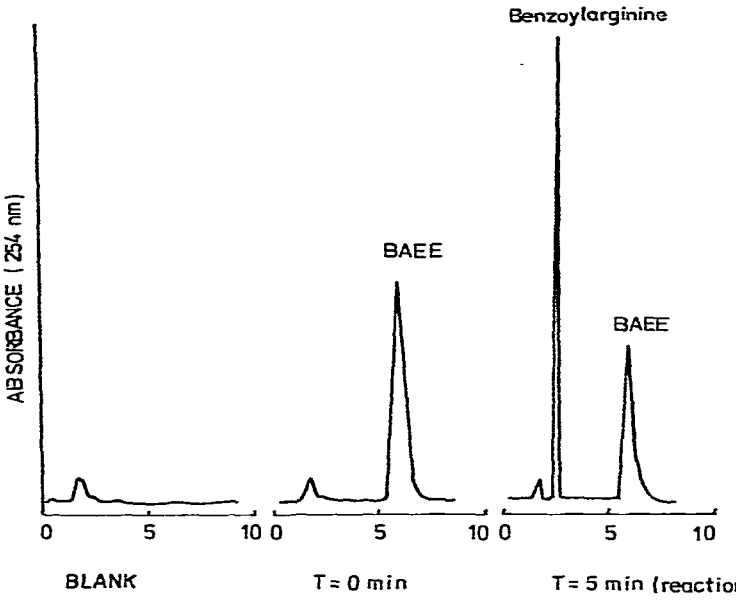

Fig. 1. HPLC profile of papain-catalysed BAEE hydrolysis product. Concitions: column, μBondapak CN; eluent, 0.05 *M* ammonium acetate–methanol (85:15); flow-rate, 2.0 ml/min; detector, 254 nm at ambient temperature.

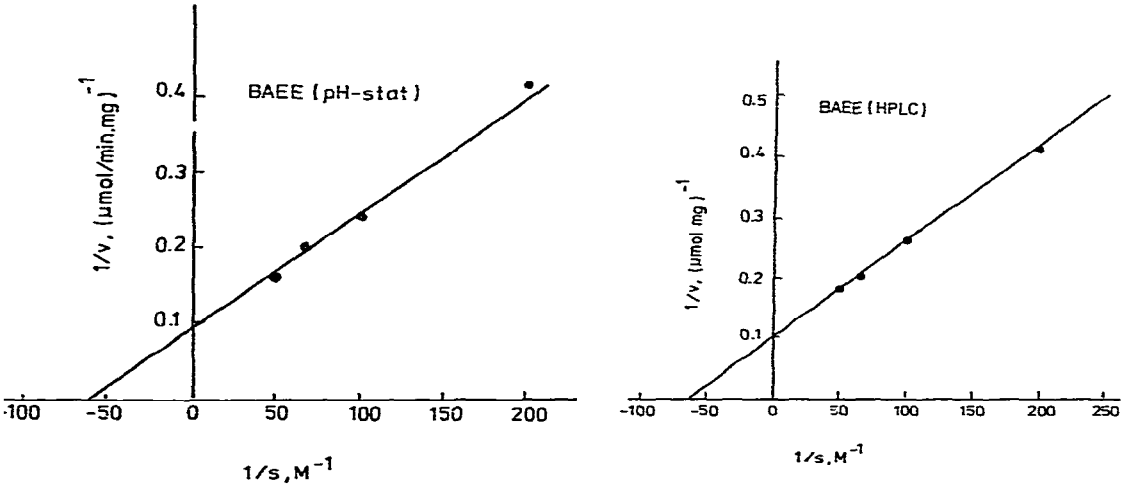

Fig. 2. Double reciprocal plot of papain for BAEE substrate using the pH-stat method. K_m = 17.14 m*M*; V_{max} = 11.10 μmole/min · mg. s = Substrate concentration; v = initial velocity.

Fig. 3. Double reciprocal plot of papain for BAEE substrate using the HPLC method. K_m = 16.67 m*M*; V_{max} = 10.57 μmole/min · mg. s = Substrate concentration; v = initial velocity.

mined by HPLC in the non-aque9us phase (Table I, Figs. 4–6). The benzyloxycarbonyl-N-protected group is more hydrophobic than the butyloxycarbonyl-N-protected group. As papain favours the bulky hydrophobic group[12], it may be predicted that papain has a greater affinity towards N-Cbz-D,L-Phe-OCH_3 and N-Cbz-D,L-Met-

TABLE I

KINETIC PARAMETERS OF PAPAIN FOR VARIOUS SUBSTRATES, N-ACYL-D,L-AMINO ACID METHYL ESTERS, MEASURED BY HPLC

Substrate	K_m *(mM)*	V_{max} *(μmole/min·mg)*
Cbz-D,L-Phe-OCH_3	13.1	0.74
Boc-D,L-Phe-OCH_3	36.3	2.50
Cbz-D,L-Met-OCH_3	13.5	4.60

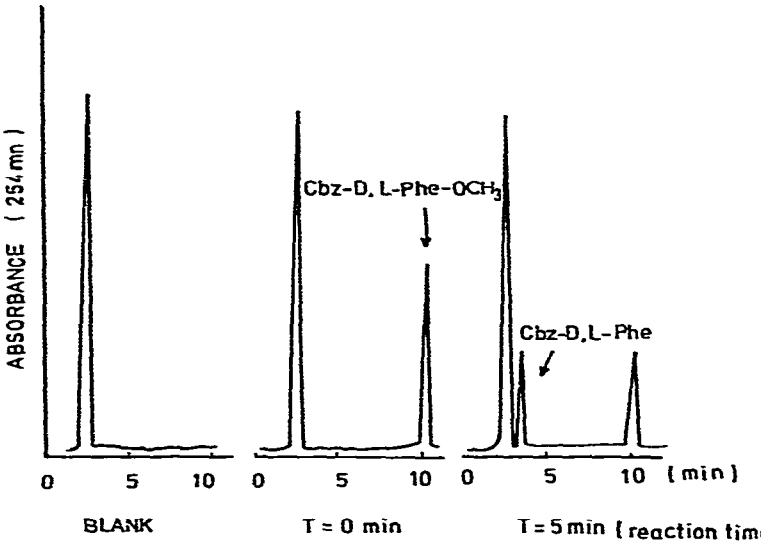

Fig. 4. HPLC profile of papain-catalysed hydrolysis of Cbz-D,L-Phe-OCH_3. Conditions: column, μBondapak C_{18}; eluent, 0.05 *M* ammonium acetate–methanol (50:50); flow-rate, 2.0 ml/min; detector, 254 nm at ambient temperature.

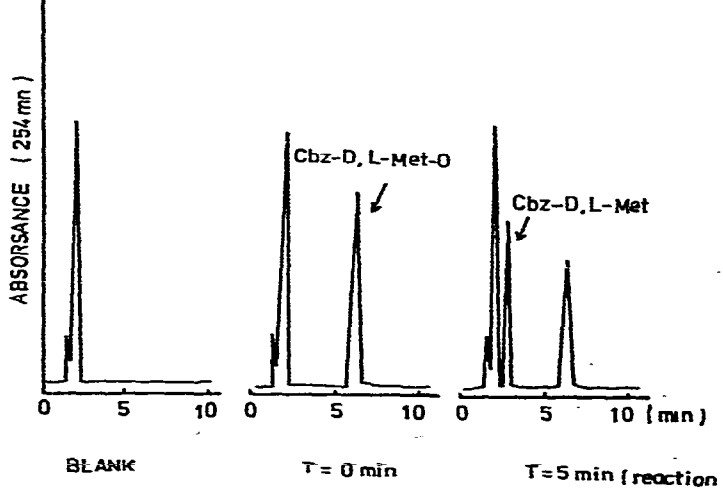

Fig. 5. HPLC profile of papain-catalysed hydrolysis of Cbz-D,L-Met-OCH_3. Conditions: column, μBondapak C_{18}; eluent, 0.05 *M* ammonium acetate–methanol (55:45); flow-rate, 2.0 ml/min; detector, 254 nm at ambient temperature.

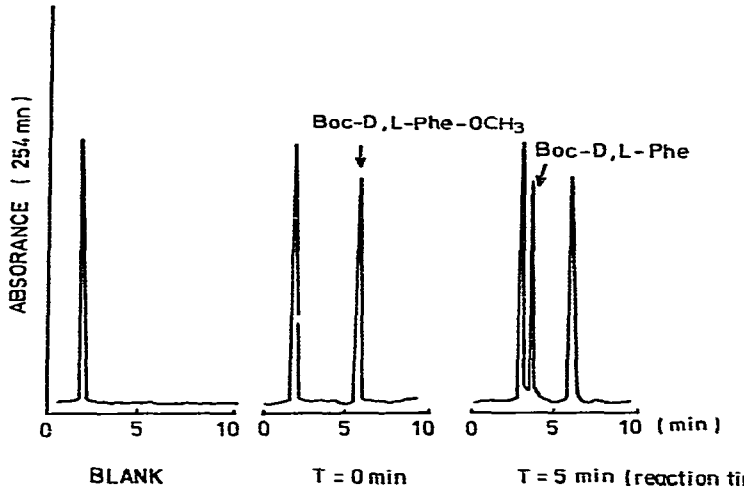

Fig. 6. HPLC profile of papain-catalysed hydrolysis of Boc-D,L-Phe-OCH_3. Conditions: column, μBondapak C_{18}; eluent, 0.05 *M* ammonium acetate–methanol (55:45); flow-rate, 2.0 ml/min; detector, 254 nm at ambient temperature.

OCH_3 than N-Boc-D,L-Phe-OCH_3. This prediction is supported by the K_m values in Table I.

In conclusion, the HPLC method has two advantages over the conventional pH-stat method for enzyme assay: (1) If the enzyme assay is carried out at high concentrations of organic solvent, pH-stat method will be unsuitable for the non-aqueous phase. HPLC overcomes this drawback. (2) In the HPLC method, the reactants and products of the enzyme-catalysed reaction can be monitored simultaneously.

REFERENCES

1 M. Zakaria and P. R. Brown, *J. Chromatogr.*, 226 (1981) 267.
2 P. R. Brown and A. M. Krstulovic, *Anal. Biochem.*, 99 (1979) 1.
3 A. M. Krstulovic, P. R. Brown and D. M. Rosie, *Anal. Chem.*, 49 (1977) 2237.
4 R. A. Hartwick, A. Jeffries, A. Krstulovic, and P. R. Brown, *J. Chromatogr. Sci.*, 16 (1978) 427.
5 C. H. Wong, M. F. Ho and K. T. Wang, *J. Org. Chem.*, 43 (1978) 3604.
6 J. E. Abernethyl, E. Albano, and J. Comyns, *J. Org. Chem.*, 36 (1971) 1580.
7 A. I. Vogel, *A Textbook of Practical Organic Chemistry*, Longmans, New York, 3rd ed., 1956, p. 970.
8 G. A. Fletcher and J. H. Jones, *Int. J. Peptide Protein Res.*, 4 (1972) 347.
9 A. I. Vogel, *A Textbook of Practical Organic Chemistry*, Longmans, New York, 3rd ed., 1956, p. 973.
10 J. Nagasawa, K. Kuroiwa, K. Narita and Y. Isowa, *Bull. Chem. Soc. Jap.*, 46 (1973) 1269.
11 R. Arnon *Methods Enzymol.*, 19 (1970) 226.
12 A. N. Glazer and E. L. Smith, in P. D. Boyer (Editor), *The Enzyme*, Vol. III, Academic Press, New York, London, 1971, p. 501.