Journal of Chromatography, 520 (1990) 333–338
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 1878

High-performance liquid chromatographic method for the study of solvent effects on the peptidase and esterase activity of thermitase

STEFFI DUDEK
Institute of Biochemistry, Medical Faculty, Martin-Luther University, DDR-4010 Halle (G.D.R.)
SIEGLINDE FRIEBE
Section Biotechnikum, Martin-Luther University, DDR-4010 Halle (G.D.R.)
and
PETER HERMANN*
Institute of Biochemistry, Medical Faculty, Martin-Luther University, DDR-4010 Halle (G.D.R.)

ABSTRACT

The high-performance liquid chromatographic separation of enzymatic degradation products of a multi-functional peptide substrate under isocratic conditions is described. This technique was applied to the study of solvent effects on the peptidase and esterase activity of thermitase.

INTRODUCTION

Thermitase is a well characterized extracellular alkaline protease from *Thermoactinomyces vulgaris* [1]. Its substrate specificity [1–3], amino acid sequence [4], mechanism of action [1] and three-dimensional structure [5] are known.

Owing to its thermalstability and its strong regio- and stereospecificity, thermitase is applied as a reagent in C-terminal ester deprotection in peptide synthesis [6,7]. In peptides containing thermitase-sensitive peptide bonds, the low peptidase activity can give rise to undesirable peptide bond splitting during preparative enzymatic esterolysis. Preliminary results showed that the peptidase activity can be selectively suppressed by the addition of polar aprotic solvents [6,8].

To study these solvent effects in more detail, a test peptide derivative as a substrate and a suitable analytical technique are required. Benzyloxycarbonylglycylphenylalanylglycylphenylalanine methyl ester, Z–Gly–Phe–Gly–Phe–OCH_3, has all the properties needed for a test peptide: the peptide is in accord with the specificity including subsite specificity of thermitase [1,2], it has one sensitive ester bond, it has only one sensitive peptide bond (–Phe–Gly–) and all the possible degradation products have UV absorption peaks near 254 nm [9].

0021-9673/90/$03.50

EXPERIMENTAL

Materials

The solvents were distilled and dried before use. Z–Gly–Phe–Gly–Phe–OCH_3, Z–Gly–Phe–Gly–Phe–OH, Z–Gly–Phe–OH and H–Gly–Phe–OH were prepared as described [8]. The peptides were characterized by elemental analysis, amino acid analysis, UV spectra [9], melting point, optical rotation and controlled for purity by thin-layer chromatography (TLC) in different systems and high-performance liquid chromatography (HPLC) (Fig. 1).

Thermitase was isolated from a culture medium of *Thermoactinomyces vulgaris* by a single-step adsorption procedure using porous glass [1]. The enzyme preparation was homogeneous according to polyacrylamide gel electrophoresis as described [1].

The enzyme solution (5.5 mg/ml) was stored at 4°C in 0.075 *M* ammonium acetate buffer (pH 6.0) under nitrogen. Proteolytic activity, as determined at pH 8.0 and 55°C in 25 m*M* Tris–HCl buffer using succinyl–$(Ala)_3$–*p*-nitroanilide (0.25 m*M*) as a substrate [10], was 0.6 μmol/min · mg.

Methods

HPLC was performed with a Merck-Hitachi LiChroGraph system using an L-6200 pump and a D-3000 photodiode-array detector. A 250 × 4 mm I.D. LiChroSpher 100 RP-8 (10 μm) column was used. UV absorption was detected at 254 nm. The flow-rate was 1 ml/min.

The enzyme assay was carried out as follows: 20 or 50 μl of the respective solvent containing 2 μmol of the substrate were diluted to 100 μl with 0.06 *M* phosphate buffer (pH 7.8) and equilibrated at 55°C. The reaction was started with 25 μg of thermitase. Parallels were incubated for different time intervals, then diluted with the acidic eluent, heated for 10 min at 70°C and applied (20 μl) to the column.

The degradation products were identified using established methods of peptide analysis [11]. The compounds were separated and isolated from samples of semi-preparative incubation mixtures performed in 20% dimethylformamide (DMF) after 5 and 60 min (see Fig. 3). The N-protected peptides were characterized by UV spectra [9], amino acid analysis, C-terminal amino acid by carboxypeptidase A digestion. H–Gly–Phe–OH was identified by end-group determination (fluorodinitrobenzene technique) and amino acid analysis. All isolated degradation products were compared with authentic material using TLC on Kieselgel G plates using the systems butanol–methanol–acetic acid–water (4:4:1:2), chloroform–methanol (88:12) and cyclohexane–ethyl acetate (1:1) and reversed-phase HPLC with photodiode-array detection including UV spectra comparison.

In incubation mixtures containing low concentrations of DMF, dimethyl sulphoxide (DMSO), dioxane or 1,4-butanediol, part of the substrate is not dissolved. On dilution with the eluent all the material dissolves. Therefore, kinetic measurements of the activities of thermitase in these experiments were not performed.

RESULTS AND DISCUSSION

The main problem was the separation of the mixture of the substrate and its degradation products (see Fig. 1) in one analytical run under isocratic conditions. The

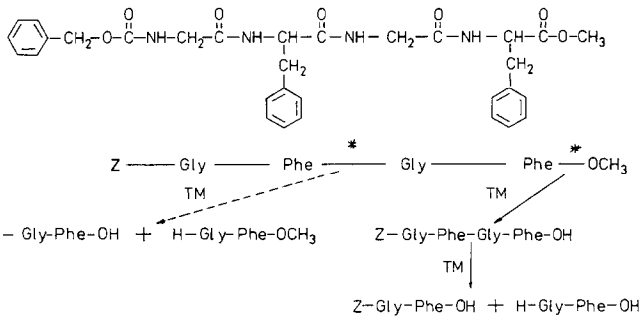

Fig. 1. Enzymatic degradation of Z–Gly–Phe–Gly–Phe–OCH_3 by thermitase (TM). Solid arrows, enzymatic attack observed; dashed arrow, enzymatic attack not observed.

substrate and the three observed degradation products, Z–Gly–Phe–Gly–Phe–OH, Z–Gly–Phe–OH and H–Gly–Phe–OH, differ markedly in their polarities.

From several systems studied, acetonitrile–water (1:1), acidified with trifluoroacetic acid (TFA) to pH 2, was selected as the mobile phase. The choice of pH 2 was of great importance for peak sharpness, chromatographic resolution and the elution order of all components (see Fig. 2). This pH guarantees the common protonated state of the carboxy groups (see Fig. 3). The use of TFA or phosphoric acid makes no difference.

Figs. 4 and 5 show the effect of 20% and 50% DMF, respectively, on the peptidase and esterase activity of thermitase. With 50% DMF the peptidase activity

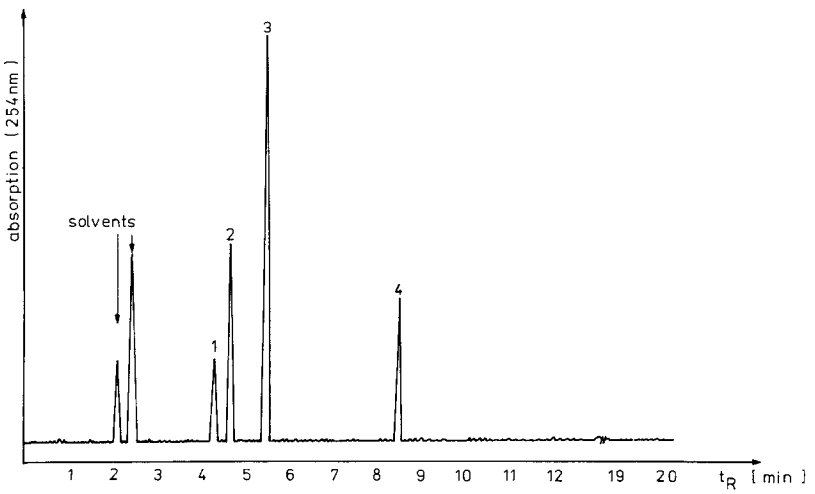

Fig. 2. Thermitase-catalysed hydrolysis of Z–Gly–Phe–Gly–Phe–OCH_3. Conditions: 20% DMF; incubation time, 10 min; otherwise as under Experimental. Peaks: 1 = H–Gly–Phe–OH (retention time, t_R = 4.34 min; capacity factor, k' = 1.17); 2 = Z–Gly–Phe–OH (t_R = 4.70 min; k' = 1.35); 3 = Z–Gly–Phe–Gly–Phe–OH (t_R = 5.40 min, k' = 1.7); 4 = Z–Gly–Phe–Gly–Phe–OCH_3 (t_R = 8.20 min, k' = 3.1). Separation factors (α): peaks 1–2 = 1.15; peaks 2–3 = 1.26; peaks 3–4 = 1.82.

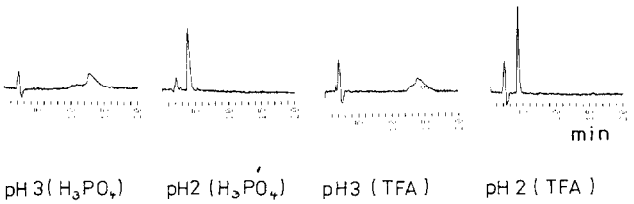

Fig. 3. Influence of pH and the acids used for pH adjustment on the chromatographic resolution and peak sharpness of H–Gly–Phe–OH. Mobile phase: acetonitrile–water (1:1); RP-8 column; flow-rate: 1.0 ml/min.

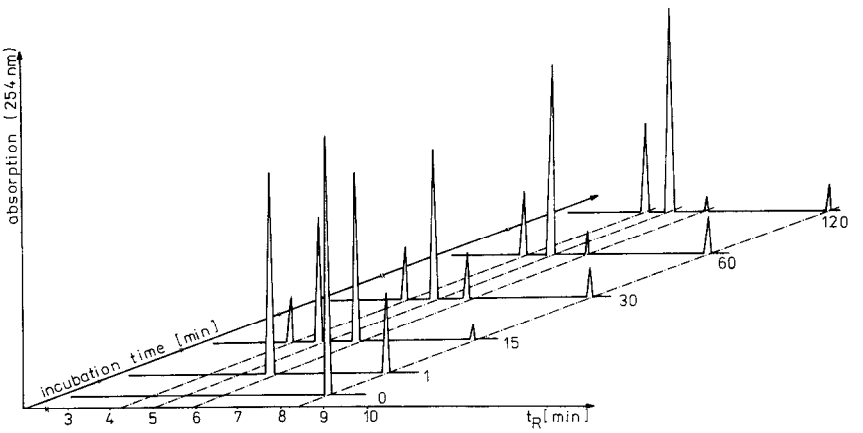

Fig. 4. Time course of the thermitase-catalysed hydrolysis of Z–Gly–Phe–Gly–Phe–OCH_3 in the presence of 20% DMF.

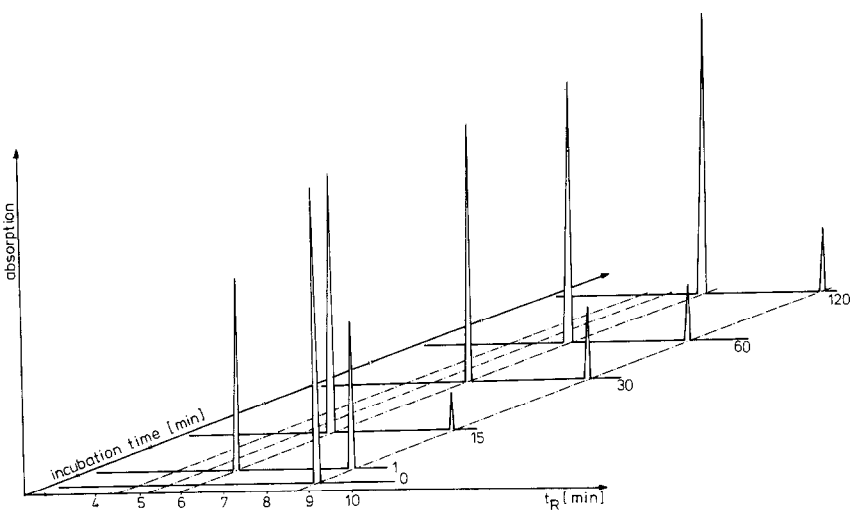

Fig. 5. Time course of the thermitase-catalysed hydrolysis of Z–Gly–Phe–Gly–Phe–OCH_3 in the presence of 50% DMF.

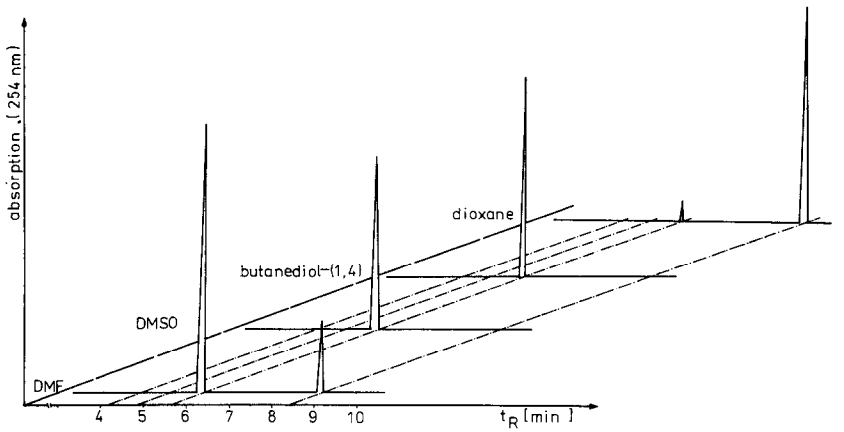

Fig. 6. Thermitase-catalysed hydrolysis of Z–Gly–Phe–Gly–Phe–OCH_3 with 50% (v/v) organic co-solvent during a 5-min incubation.

is selectively suppressed whereas the esterase activity is sufficiently high for preparative peptide synthesis. Incubations without DMF for the purpose of comparison could not be performed because of the sparingly soluble substrate.

Similar results were obtained with DMSO and dioxane. With 1,4-butanediol as a protic polar solvent, the peptidase activity was hardly influenced.

In Figs. 6 and 7 the results of the experiments are summarized with respect to brief and prolonged incubations in mixtures of 50% of the different organic solvents. From these results 50% polar aprotic solvent mixtures can be recommended for selective preparative C-terminal ester deprotection in peptide synthesis if the peptide sequence under study contains thermitase-sensitive peptide bonds. 1,4-Butanediol, otherwise recommended in enzymatic peptide synthesis [12], fails to guarantee selective ester hydrolysis. Concentrations of the polar organic solvents higher than 50% give a greater reduction of the esterase activity of thermitase.

In conclusion, HPLC has been found to be useful for the analysis of the action of different enzyme activities on a multi-functional substrate.

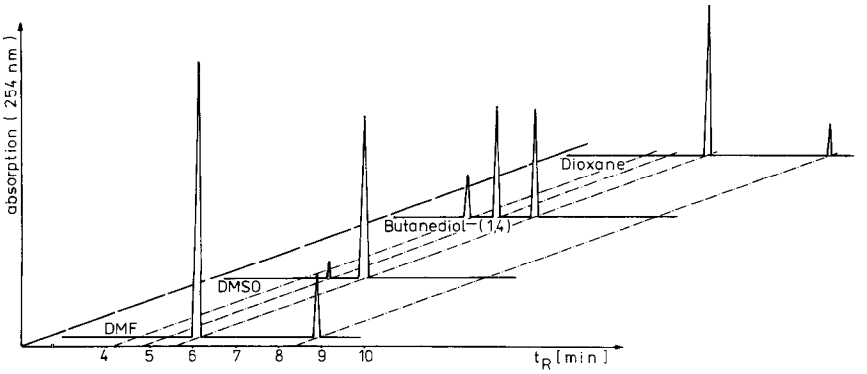

Fig. 7. Thermitase-catalysed hydrolysis of Z–Gly–Phe–Gly–Phe–OCH_3 with 50% (v/v) organic co-solvent during a 120-min incubation.

REFERENCES

1 R. Kleine, U. Rothe, U. Kettmann and H. Schelle, in V. Turk and L. J. Vitale (Editors), *Proteinases and their Inhibitors,* Mladinska Kniga, Ljubljana, 1981, p. 201.
2 D. Brömme and R. Kleine, *Curr. Microbiol.,* 11 (1984) 93.
3 D. Brömme, K. Peters, S. Fink and S. Fittkau, *Arch. Biochem. Biophys.,* 211 (1986) 439.
4 B. Meloun, M. Baudys, V. Kostka, G. Hausdorf, C. Frömmel and W. E. Höhne, *FEBS Lett.,* 183 (1985) 195.
5 C. Frömmel and C. Sander, *Proteins,* 5 (1989) 22.
6 P. Hermann and L. Salewski, in K. Blaha and P. Malon (Editors), *Peptides 1982,* Walter de Gruyter, Berlin, 1983, p. 399.
7 P. Hermann, in B. Wilhelmi (Editor), *Chemie und Biologie von Peptidwirkstoffen,* Wissenschaftliche Beiträge der Friedrich-Schiller Universität, Jena, 1986, p. 101.
8 I. Schön and P. Hermann, in B. Penke and A. Török (Editors), *Peptides,* Walter de Gruyter, Berlin, 1988, p. 79.
9 K. Vogler, J. Würsch, R. O. Studer and P. Lanz, *Chimia,* 14 (1960) 379.
10 D. Brömme and S. Fittkau, *Biomed. Biochim. Acta,* 44 (1985) 1089.
11 T. Devenyi and J. Gergely, *Amino Acids, Peptides and Proteins. Biochemical and Immunochemical Techniques in Protein Chemistry,* Elsevier, Amsterdam, 1974, pp. 163, 166, 170, 238 and 266.
12 V. S. Ganu and J. D. Glass, in K. Blaha and P. Malon (Editors), *Peptides 1982,* Walter de Gruyter, Berlin, 1983, p. 403.