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Note

Chromogenic substrates for proteinases

Purification by chromatography on lipophilic Sephadex

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Routine assays of proteolytic enzymes rely heavily on the use of chromogenic substrates. In their simplest form these substrates are esters or amides of N-acyl amino acids[1,2]. Amide substrates are less suitable than esters for the assay of serine enzymes since, in general, they are hydrolysed more slowly[3]. The most widely available chromogenic ester substrates are those derived from 4-nitrophenol, since these compounds are important intermediates in peptide synthesis[4]. 4-Nitrophenyl esters are excellent substrates for thiol proteinases, which have maximum activity at mildly acidic pH values[5], but the 4-nitrophenyl esters hydrolyse spontaneously at alkaline pH values[6], so that, for serine enzymes, the assays may have to be made at pH values well below the pH optimum[7].

Naptholic esters are less susceptible to alkaline hydrolysis and Ravin *et al.*[8] developed a colorimetric assay for chymotrypsin in which the 2-naphthol released from benzoyl DL-phenylalanine 2-naphthyl ester was converted to an insoluble azo dye, and the dye extracted into ethyl acetate. The liberation of naphthols may also be detected fluorometrically[9,10] or by spectrophotometry[11]. The azo dye method would be ideal for routine spectrophotometric assays if the dye could be solubilized in aqueous solutions. This problem may be overcome by diazo coupling in the presence of a suitable detergent[12,13].

2-Naphthyl esters of α-N-acyl L-amino acids are not available commercially, although they may be synthesised under conditions which retain the L-optical configuration[14]. We wished to prepare small quantities (approx. 100 mg) of materials to test as substrates for two proteinases in human spleen which degrade azo-casein at neutral pH[15]. Although a high yield of product was desirable, the primary need was the absence of contaminating 2-naphthol. The application of chromatography on Sephadex LH-20 seemed promising from the results of Streuli[16,17] who observed that during the chromatography of aromatic molecules those with a structure allowing hydrogen bonding to the matrix were strongly retarded. Since esters of N-acetyl aromatic amino acids are not retained during chromatography on Sephadex LH-20 in methanol[18] it seemed probable that efficient separation would be achieved.

EXPERIMENTAL*

Materials and analytical methods

Sephadex LH-20 was obtained from Pharmacia (London, Great Britain). N-Acyl amino acids were obtained from Bachem (Liestal, Switzerland). Brij 35, calcium hydride, dicyclohexylcarbodiimide, 2-naphthol and molecular sieve 3A were obtained from BDH (Poole, Great Britain). Fast Garnet GBC salt, naphthol AS and naphthol AS-D were obtained from Sigma (Kingston-upon-Thames, Great Britain). 7-Hydroxy-4-methylcoumarin was obtained from Koch-Light (Colnbrook, Great Britain).

2-Naphthol was recrystallised from benzene. Naphthol AS was recrystallised from tetrahydrofuran–dimethylformamide (5:1, v/v). Naphthol AS-D was twice crystallised from tetrahydrofuran–ethanol (1:1, v/v). 7-Hydroxy-4-methylcoumarin was recrystallised from ethanol. Acetonitrile was distilled and the fraction b.p. 81–82° collected and stored over molecular sieve 3A. Dimethylformamide was purified by the method of Green *et al.*[19], omitting the vacuum redistillation. Tetrahydrofuran was distilled and the fraction b.p. 66–67° collected and stored in darkness over calcium hydride.

Melting points (uncorrected) were determined with an MF-370 apparatus supplied by A. Gallenkamp & Co. (London, Great Britain). The elemental analyses were carried out by Dr. F. B. Strauss, Microanalytical Laboratory, Oxford, Great Britain.

Synthesis and chromatography

Phenolic esters were synthesised by the carbodiimide method in pyridine[14]. Column chromatography was performed at 21° in glass apparatus with PTFE connecting tubing. The procedure is described in detail for *tert.*-butyloxycarbonyl-L-alanine 2-naphthyl ester (Boc-Ala-2-ONap).

tert.-Butyloxycarbonyl-L-alanine (100 mg, 0.53 mmol) and 2-naphthol (87 mg, 0.60 mmol) were dissolved in pyridine (0.5 ml) at 0°. Dicyclohexylcarbodiimide (124 mg, 0.60 mmol) was added with stirring and the mixture allowed to warm to 21° overnight. Acetonitrile (5 ml) was added and the precipitated dicyclohexylurea removed by filtration. The filtrate was evaporated to dryness at 35° *in vacuo*, and the residue dissolved in ethanol (1 ml) and applied to a column (1.5 cm × 80 cm, 140 ml) of Sephadex LH-20 in ethanol. The column was eluted at 25 ml/h and 2-ml fractions were collected. The column effluent was monitored at 300 nm in a 0.1-cm flow cell. Portions (2 μl) of each fraction were spotted on to a silica gel sheet and sprayed with Fast Garnet GBC (0.5 mg/ml) in ethanol–water (7:3, v/v). Portions (10 μl) of fractions containing Boc-Ala-2-ONap (see Results and Discussion) were mixed with 5 *M* NaOH (25 μl) and stood at 21° for 10 min. 1.2 *M* HCl (0.1 ml) and 1 *M* sodium acetate pH 5.5 (0.1 ml) were added, followed by Fast Garnet GBC solution (0.2 ml) as above. Control experiments were made on samples not subjected to alkaline hydrolysis. Fractions containing Boc-Ala-2-ONap were combined and evaporated to dryness at 35° *in vacuo* to give a colourless solid (148 mg, 89%).

* Abbreviations used: Ac = acetyl; Boc = *tert.*-butyloxycarbonyl; Z = benzyloxycarbonyl; naphthol AS = 2-hydroxy-3-naphthoic acid anilide; naphthol AS-D = 2-hydroxy-3-naphthoic acid *p*-toluidide; -2-ONap = 2-naphthyl ester; -2-ONapAS = 2-naphthyl AS ester; -2-ONapAS-D = 2-naphthyl AS-D ester; -7-OHmc = 7-(4-methyl)coumarinyl ester; -2-SNap = 2-thionaphthyl ester.

The naphthol content of the product was estimated by alkaline hydrolysis. Standard solutions of Boc-Ala-2-ONap (0.156*M*) and 2-naphthol (0.152*M*) were prepared in ethanol. Boc-Ala-2-ONap solution (25 μl) was mixed with 5 *M* NaOH (0.1 ml) and left at 21° for 15 min. 1.2 *M* HCl (0.4 ml) and ethanol (3.5 ml) were added. Unhydrolysed controls and 2-naphthol standards were prepared by mixing portions (25 μl) of each solution with water (0.5 ml) and ethanol (3.5 ml). Portions (5–50 μl) of these solutions were added to Fast Garnet GBC (0.05 mg/ml) in 0.5 *M* sodium acetate (pH 5.5) containing 2% (w/v) Brij 35 (2 ml). The absorbances at 520 nm were read after 45 min.

RESULTS AND DISCUSSION

Chromatography of the partially purified coupling mixture on Sephadex LH-20 in ethanol resulted in two well-resolved peaks absorbing at 300 nm, as shown in Fig. 1. When the fractions were tested for 2-naphthol, by drying portions on a silica gel sheet and spraying with Fast Garnet GBC solution, the first peak, fractions 41–49, gave a weak purple colour, but fractions 56–64 stained intensely. This suggested that Boc-Ala-2-ONap was eluted before 2-naphthol, but that some decomposition of the ester had occurred. However, fractions 42–48 were found to be free of 2-naphthol when tested under conditions in which approx. 0.1% contamination would have been detected. It is possible that hydrolysis occurs more readily on the hydrophilic silica gel layer, since Z-Ala-7-OHmc showed a similar effect.

Portions of a solution of the material isolated from fractions 41–49 were treated with alkali and the liberated 2-naphthol was coupled with Fast Garnet GBC[13]. The absorbances at 520 nm were compared with those given by portions of a 2-naphthol solution of known concentration. This comparison showed that chromatography on Sephadex LH-20 in ethanol gave a product which contained 95% Boc-Ala-2-ONap. The yield of 2-naphthyl ester was 84%.

The method was used successfully in the synthesis and purification of a number of other compounds, although modifications to the method described for Boc-Ala-

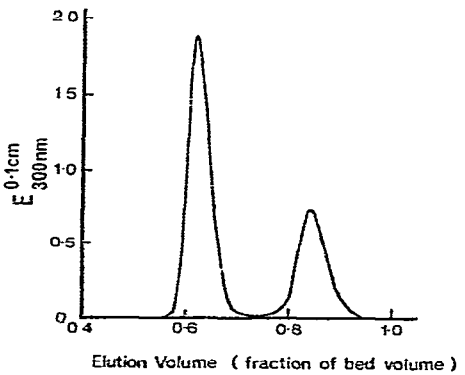

Fig. 1. A preparation of Boc-Ala-2-ONap (see text for details) was applied to a column (1.5 cm × 80 cm, 140 ml) of Sephadex LH-20 in ethanol. The column was eluted with ethanol at 25 ml/h and 2-ml fractions were collected. The absorbance of the effluent was monitored at 300 nm in a 0.1-cm flow cell.

TABLE I

ANALYTICAL DATA FOR SOME N-ACYL AMINO ACID PHENOLIC ESTERS

Compound	*M.p. (°C)*	*Formula*	*Elemental composition (%)*	
			Calculated	*Found*
Boc-Ala-2-ONap	102–103	$C_{18}H_{21}NO_4$	C 68.55	68.80
			H 6.71	6.65
			N 4.44	4.50
Z-Ala-2-ONap	123–124	$C_{21}H_{19}NO_4$	C 72.19	72.23
			H 5.48	5.47
			N 4.01	4.22
Z-Phe-2-ONap	109	$C_{27}H_{23}NO_4$	C 76.22	76.00
			H 5.45	5.52
			N 3.29	3.36
Z-Ala-7-OHmc	123–124	$C_{27}H_{19}NO_5$	C 66.14	66.03
			H 5.02	5.16
			N 3.67	3.79

2-ONap were sometimes necessary. In all cases reaction in the minimum of solvent with a 10% excess of both the phenol and dicyclohexylcarbodiimide gave an acceptable yield of ester (80% or more) after reaction for approx. 20 h. Naphthol AS and naphthol AS-D were rather insoluble in pyridine, and esters derived from these compounds were synthesised in pyridine–dimethylformamide (1:1, v/v).

It was observed that Z-Ala-2-ONapAS decomposed in ethanol, and to avoid the risk of ester exchange during chromatography, columns have been run in acetonitrile, tetrahydrofuran or acetic acid. In solvents other than ethanol, samples from each fraction were spotted on to silica gel sheets and sprayed with 0.2 *M* Tris–HCl (pH 9) in ethanol–water (7:3, v/v), to hydrolyse any ester present. After drying at 120° the sheets were sprayed with the Fast Garnet GBC solution.

Table I gives the melting points, formulae and calculated elemental compositions of a number of compounds prepared by this method. In each case the identity was confirmed by elemental analysis. In addition to the compounds analysed, the method was shown to be suitable for the preparation and purification of the following reagents, the chromatographic solvent being shown in brackets: Z-Ala-2-ONapAS (acetic acid); Z-Ala-2-ONapAS-D (acetonitrile); Boc-Ala-7-OHmc (tetrahydrofuran); Z-Ala-2-SNap (acetonitrile); Ac-Phe-2-ONap (tetrahydrofuran).

In conclusion, chromatography on lipophilic Sephadex allows the rapid and convenient purification of small quantities of chromogenic substrates derived from N-acyl amino acids. The products are not contaminated by the chromophore, and good yields are obtained by avoiding the usual losses associated with recrystallization.

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