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PRE-COLUMN LABELLING FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS WITH 7-FLUORO-4-NITROBENZO-2-OXA-1,3-DIAZOLE AND ITS APPLICATION TO PROTEIN HYDROLYSATES*

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SUMMARY

A high-performance liquid chromatographic (HPLC) method for the determination for minute amounts of eighteen amino acids of the presumed components of protein hydrolysates is presented. The method consists of derivatization of the amino acids with a new fluorigenic reagent, 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F), in ethanol–0.1 *M* phosphate buffer (pH 8.0) (50:50) at 60°C for 1 min, separation of the resultant fluorophores, including ε-aminocaproic acid as an internal standard, on a reversed-phase column (μBondapak C_{18}) by (i) isocratic elution by solvent A [methanol–tetrahydrofuran (THF) in 0.1 *M* phosphate buffer (pH 6.0) (3.75:1.6:94.65)] for 24 min, (ii) a linear gradient of 100% solvent A to 100% solvent B [methanol–THF in 0.1 *M* phosphate buffer (pH 6.0) (25:15:60)] over 30 min, (iii) isocratic elution using solvent B for 6 min and (iv) an isocratic elution by solvent C [methanol–water (40:60)] for 12 min, and detection at 530 nm with excitation at 470 nm. The detection limit for each amino acid is *ca.* 10 fmol. Profile analysis was achieved for *ca.* 1.5 μg of protein hydrolysates (rabbit pyruvate kinase-M_1, rabbit aldolase A and papain) in the final pre-column labelling reaction mixture for HPLC.

INTRODUCTION

In order to determine small amounts of amino acids, such as in protein or peptide hydrolysates, a sensitive method is mandatory. Fluorimetry is valid for such a use. However, the popular fluorigenic reagents, fluorescamine[1] and *o*-phthalaldehyde (OPA)[2], are not suitable for such a purpose because they are not reactive to secondary amino acids such as Pro and Hyp. Although conversion of secondary amino acids into primary amines using N-chlorosuccinimide prior to the fluorigenic reaction has been developed[3], the excess of the latter reagent required obstructs the fluorescence yield of the generated fluorophores.

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7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)[4] has recently been extensively used for the detection and determination of secondary amino acids by pre-column labelling[5,6] or a post-column reaction technique[7]. Ahnoff *et al.*[6] showed that NBD-Cl is less reactive to amino acids having primary amino groups than hydroxide or methoxide anions in the reaction medium, so that solvolysis occurred before the reaction with the amino acids having primary amino groups was complete. In the case of Lys, which has an α-amino group plus ε-amino group, the reaction process is rather complex. Thus two fluorescent products for Lys were obtained which might be mono- and bis-derivatives.

Previously[8,9], we reported that 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F), presumably a more reactive homologue of NBD-Cl, proved to be more reactive to the secondary amino acids than NBD-Cl or NBD-Br, so that even amino acids having primary amino groups, such as Asp, Glu, Ser, Gly, Thr and Ala, could be derivatized simultaneously with Pro and Hyp at pH 8.0 at 60°C for 2.5 min. The derivatives were separated and detected at 1 pmol level in high-performance liquid chromatography (HPLC).

In this paper, we describe HPLC separation and determination of eighteen, rather than eight in the previous paper[9], amino acid standards derivatized with NBD-F. A profile analysis of amino acids in protein hydrolysates is also reported.

EXPERIMENTAL

Materials

NBD-F was synthesized by the method of Nunno *et al.*[10]. Amino acids were purchased from Kyowa Hakko Co., Tokyo, Japan. All other chemicals were of analytical reagent grade. Methanol and tetrahydrofuran (THF) were of HPLC grade (Nakarai Kagaku Co., Tokyo, Japan). Water was deionized and doubly distilled. The pH of the borate buffer (Na^+, 0.1 *M*, pH 8.0) was adjusted with 0.1 *M* hydrochloric acid. The phosphate buffers (pH 6.0–7.5) used as mobile phases, prepared with 0.1 *M* NaH_2PO_4 and 0.1 *M* Na_2HPO_4, were filtered through Toyo No. 6 filter-paper (Toyo Roshi, Tokyo) prior to use.

Rabbit pyruvate kinase-M_1 (E.C. 2.7.1.40), rabbit aldolase A and papain (E.C. 3.4.22.2) which were used as standard proteins were hydrolysed in the usual way with 6 *M* hydrochloric acid at 110°C for 24 h in evacuated sealed tubes. The lyophilysed hydrolysates were kindly donated by Dr. Noboru Nakai of Fukui Medical School.

HPLC

For the isocratic elution studies, a Model 6000A pump equipped with a U6K Universal Injector (Waters Assoc., Milford, MA, U.S.A.) was employed. For the gradient elution studies, two Model 6000A pumps, controlled by a Model 660 solvent programmer (Waters Assoc.) were used. A guard column of Bondapak C_{18}–Corasil (20 × 3.9 mm) and a main column of μBondapak C_{18} (300 × 3.9 mm, 10 μm) connected to the former column were used. All eluting solvents were filtered and degassed prior to use. The flow-rate was 2.0 ml/min. The column temperature was ambient. The void volume of the column was measured with NBD–Asp as a marker under elution with 100% methanol. A Hitachi 560-10S spectrofluorimeter equipped with a 18 μl flow cell was used with an excitation wavelength of 470 nm and an emission at 530 nm.

Derivatization procedure

To a 500-μl conical tube were added 10 μl of a mixed amino acid standards solution (500 pmol each) and 10 μl of borate buffer (0.1 *M*, pH 8.0). To this solution 20 μl of NBD-F (50 m*M* in ethanol, freshly prepared) were added and the tube was capped and covered with aluminium foil. The vessel was then heated at 60°C for 1 min. After cooling on ice–water, 460 μl of 0.005 *M* hydrochloric acid were added to the reaction mixture. A 10-μl volume of the solution was injected onto the column. Ten 1-μl aliquots of protein hydrolysates (*ca.* 15 μg each) mentioned under *Materials*, dissolved in 100 μl of water containing ε-aminocaproic acid (10 nmol), were derivatized and treated as for the standard amino acids.

RESULTS

Optimization of the derivatization

In the preceding paper[8], we reported that the rate of formation of the fluorophores derived from the reaction of amino acids with NBD-F increased with increasing volume of organic solvents, pH of buffer and reaction temperature. Therefore, in a previous paper[9], the reaction for derivatization prior to HPLC was performed in 50% ethanol at pH 8.0 and 60°C for 2.5 min. In this experiment, to prevent degradation of the fluorophores and to decrease the rate of hydrolysis of NBD-F to 7-hydroxy-4-nitrobenzo-2-oxa-1,3-diazole (NBD-OH), which interferes with the peaks of NBD–Gly and –Arg, milder conditions (in 50% ethanol at pH 8.0 and 60°C for 1 min) were selected.

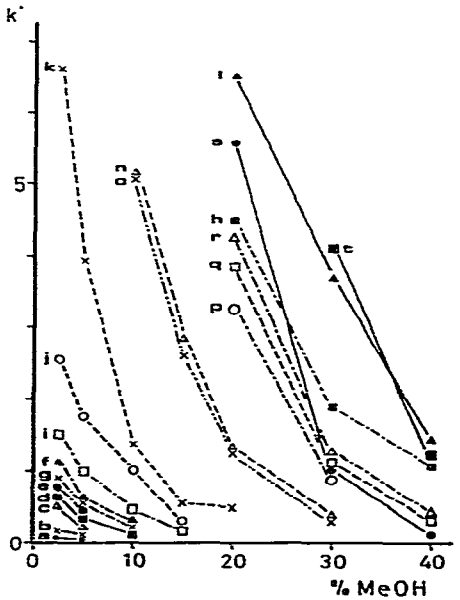

Fig. 1. Plots of k' *vs.* concentration of methanol (MeOH) for isocratic elution: a = NBD–Asp; b = NBD–Glu; c = NBD–Hyp; d = NBD–Ser; e = NBD–His; f = NBD–Gly; g = NBD-OH; h = NBD–Arg; i = NBD–Thr; j = NBD–Ala; k = NBD–Pro; l = NBD-NH_2; n = NBD–Val; o = NBD–Met; p = NBD–Ile; q = NBD–Leu; r = NBD–Phe; s = NBD–Lys; t = NBD–Tyr.

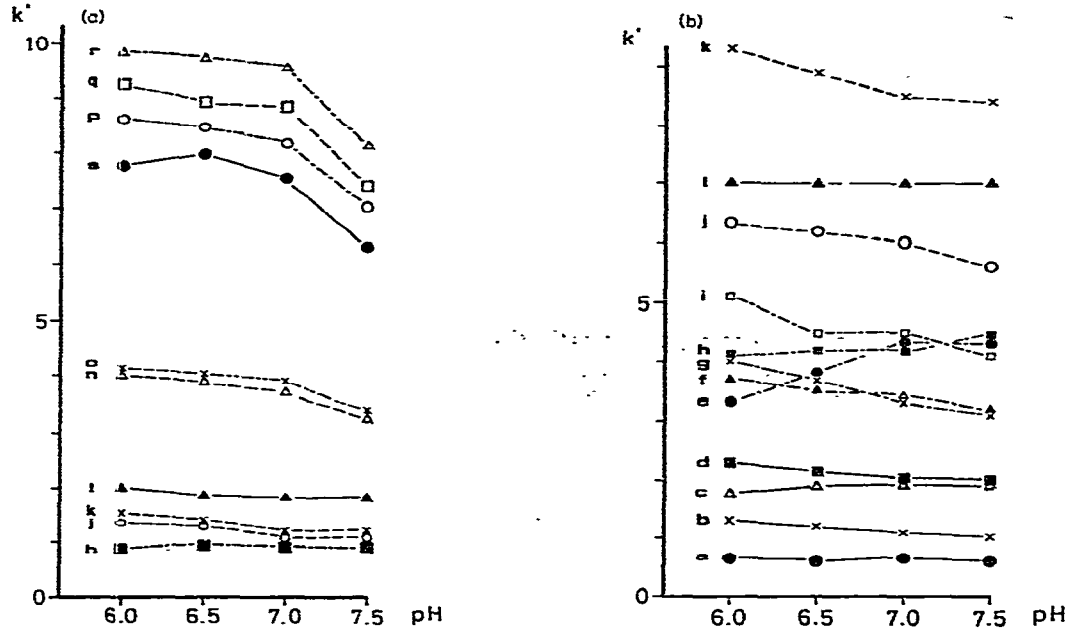

Fig. 2. Plots of k' *vs.* pH of a 0.1 *M* phosphate buffer for isocratic elution with 40% (a) or 20% (b) methanol. Abbreviations are as in Fig. 1.

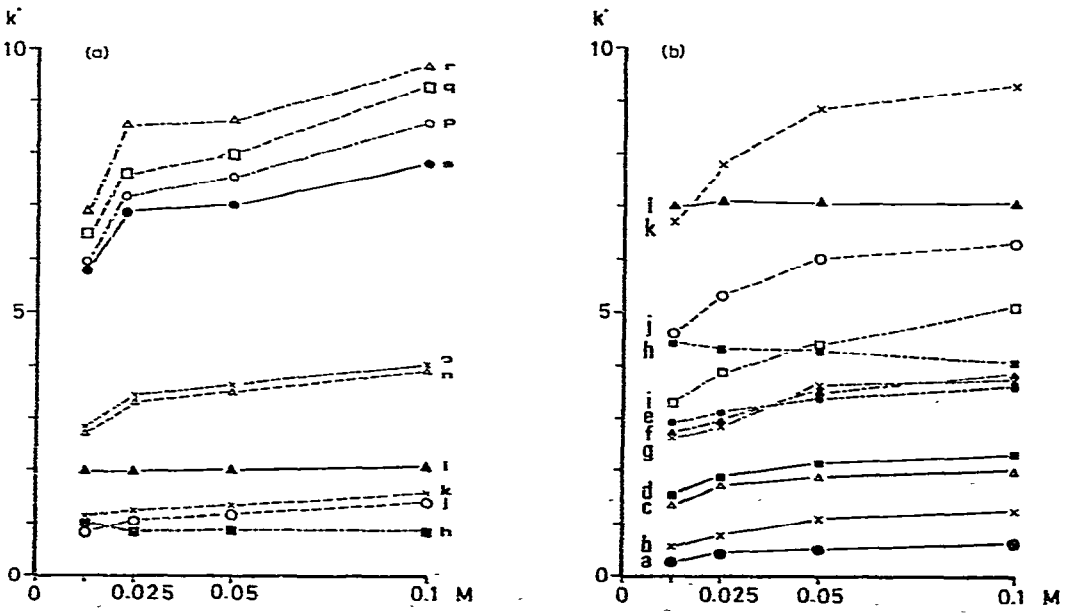

Fig. 3. Plot of k' *vs.* concentration of the phosphate buffer at pH 6.0 for isocratic elution with 40% (a) or 20% (b) methanol. Abbreviations are as in Fig. 1.

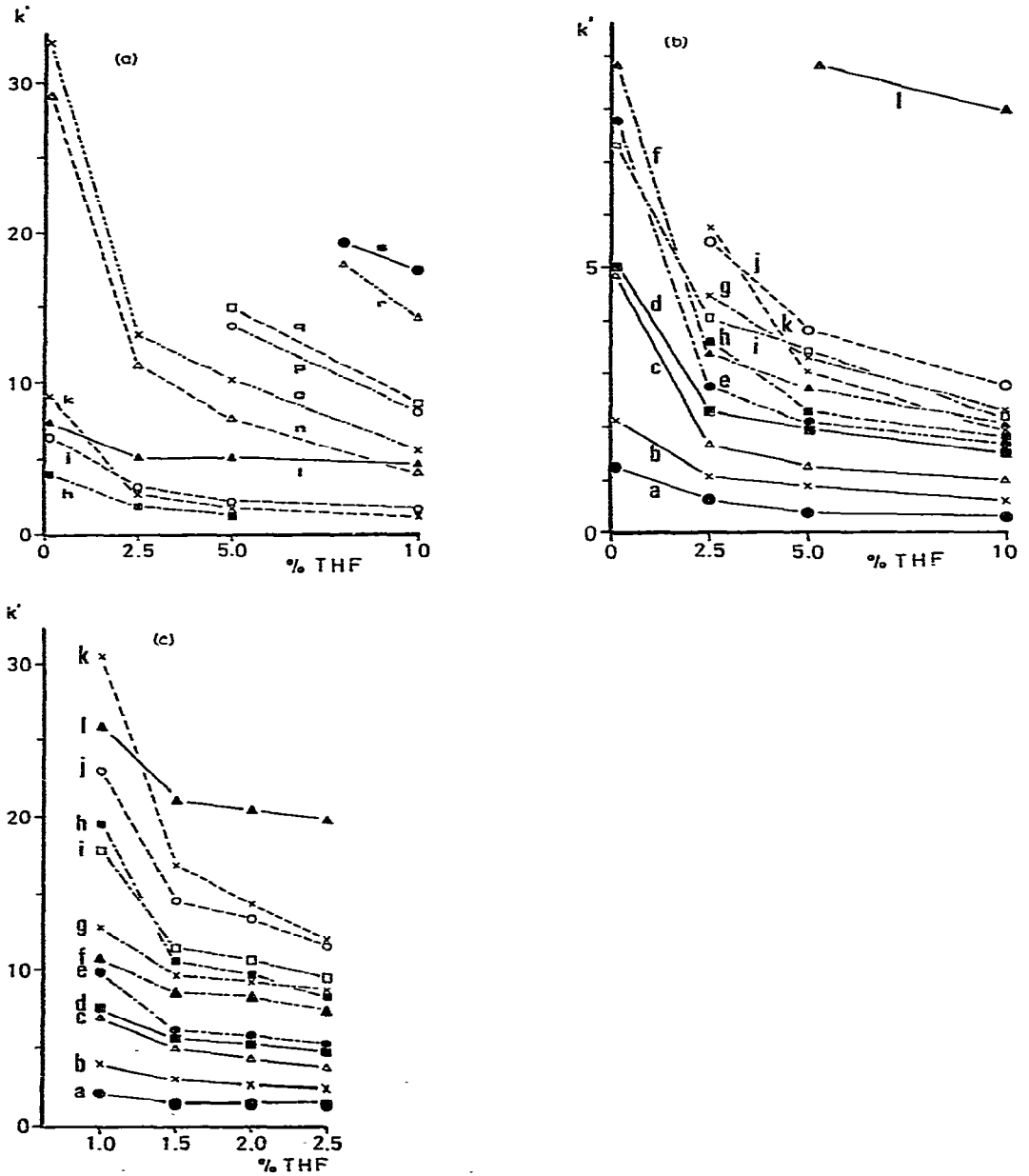

Fig. 4. Plots of k' *vs.* concentration of tetrahydrofuran for isocratic elution with 20% (a), 10% (b) or 3.75% (c) methanol in 0.1 *M* phosphate buffer at pH 6.0. Abbreviations are as in Fig. 1.

Separation of NBD–amino acid derivatives

In reversed-phase HPLC, methanol is commonly used as an eluent. In this experiment, separation of NBD–amino acids was also studied using various concentrations of methanol in water. As expected, the capacity factor (k') of all the amino acid derivatives increased as the concentration of methanol decreased (Fig. 1). How-

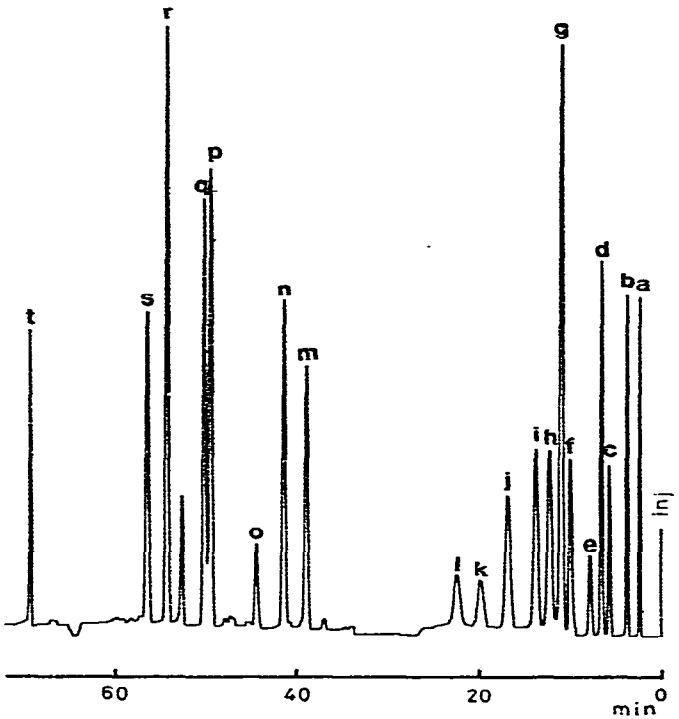

Fig. 5. Elution profile of amino acid standards derivatized by reaction with NBD-F. Each peak represents 10 pmoles. Conditions: solvent A, methanol–THF–0.1 *M* phosphate buffer (pH 6.0) (3.75:1.6:94.65); solvent B, methanol–THF–0.1 *M* phosphate buffer (pH 6.0) (25:15:60); solvent C, 40% methanol in water; gradient programme, 100% A for 24 min, linear step to 100% B over 30 min, 100% B for 6 min, 100% C for 12 min; flow-rate, 2.0 ml/min. Abbreviations are as for Fig. 1, except for (m) NBD–ε-aminocaproic acid.

ever, in this simple binary mixture of solvents, the NBD–amino acid derivatives eluted earlier, such as Asp, Glu, Hyp, Ser, His, Gly and Thr, were not well separated and some tailing of peaks was also observed. In order to retain more of the fluorophores the use of buffers (pH 6.0–7.5) containing 40 (Fig. 2a) or 20% methanol (Fig. 2b) was examined. The k' values of almost all the NBD–amino acid derivatives decreased with increasing pH while the k' of NBD–Arg increased and the k' of 7-amino-4-nitrobenzo-2-oxa-1,3-diazole (NBD-NH_2), NBD–Hyp and NBD–Asp remained approximately constant over this pH range. The effect of buffer ion concentration (phosphate buffer, pH 6.0) was examined from 0.0125 to 0.10 *M* with 40 and 20% methanol (Figs. 3a and b). The k' values increased with increasing ion concentration. The effect was greater compared to that observed with change of pH. However, the k' value of NBD-NH_2 was not affected and that of NBD–Arg decreased. Curiously, NBD–Tyr would not elute using mobile phases containing buffers, while use of simple binary mixtures of methanol–water caused elution as shown in Fig. 1 (line t). Since complete separation was difficult using the binary solvent systems examined, a ternary solvent system using THF as a third modifier[11] was investigated where an efficient separation was achieved. As shown in Figs. 4a–c, THF (1.0–10%) reduced remarkably the retentions of amino acid derivatives, especially those with large k' values. On the basis of the results obtained above, the ternary solvent system with gradient elution was se-

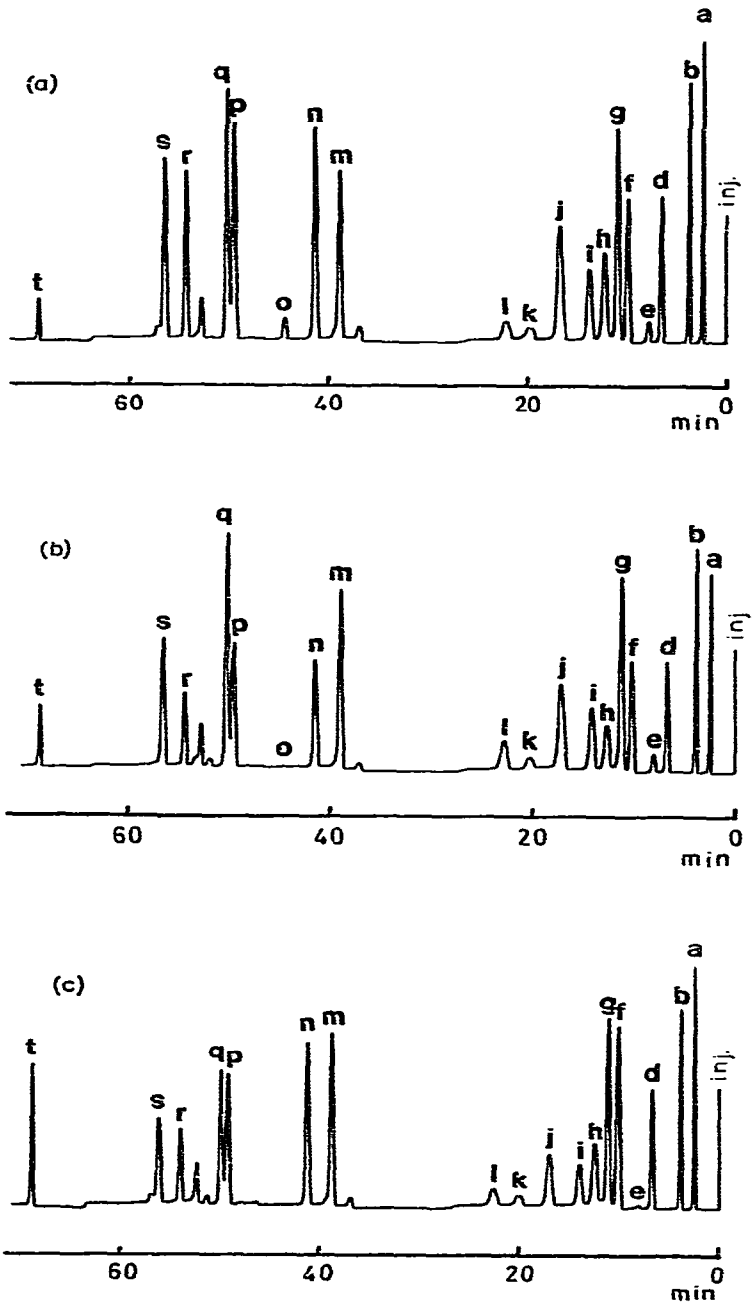

Fig. 6. Elution profiles for hydrolysates of rabbit pyruvate-M_1. (a), rabbit aldolase A (b) and papain (c). The protein (1.435 mg for a, 0.82 mg for b, 0.82 mg for c), hydrolysed with 6 *M* hydrochloric acid was diluted to a concentration of 0.146 μg/μl with an internal standard solution (100 pmol/μl). Aliquots (10 μl) of the hydrolysate were derivatized and 2% of the resulting mixture was applied onto the HPLC column. Conditions were as in Fig. 5. Abbreviations are as in Figs. 1 and 5.

TABLE I

RATIO OF AMINO ACID COMPOSITIONS OF PROTEIN HYDROLYSATES

Ratio of Ala is tentatively fixed as 1.00.

Amino acid	*Rabbit pyruvate kinase-M_1*		*Rabbit aldolase A*		*Papain*	
	NBD-F method	*Ref. 15*	*NBD-F method*	*Ref. 16*	*NBD-F method*	*Ref. 17*
Asp (+Asn)	0.71	0.83	0.63	0.67	1.22	1.34
Glu (+Gln)	0.82	0.92	0.91	0.96	1.37	1.42
Ser	0.45	0.47	0.45	0.50	0.81	0.92
His	0.21	0.21	0.27	0.26	*	0.14
Gly	0.73	0.70	0.74	0.72	1.99	2.00
Arg	0.61	0.57	0.37	0.36	0.63	0.85
Thr	0.42	0.40	0.53	0.53	0.56	0.56
Ala	1.00	1.00	1.00	1.00	1.00	1.00
Pro	0.25	0.37	0.37	0.46	0.54	0.72
Val	0.77	0.76	0.51	0.50	1.28	1.28
Met	0.29	0.29	*	0.07	0	0
Ile	0.58	0.61	0.46	0.46	0.76	0.86
Leu	0.71	0.73	0.85	0.81	0.82	0.78
Phe	0.29	0.26	0.17	0.17	0.29	0.28
Lys	0.62	0.61	0.58	0.62	0.63	0.72
Tyr	0.17	0.17	0.30	0.29	1.18	1.36

* Not determined as a measurable peak was not obtained.

lected for the separation of the NBD–amino acid derivatives. Of the several gradient shapes available, linear gradient elution proved most effective. The eluting solvents for all the NBD–amino acids were as follows; (A) 3.75% methanol–1.6% THF in 0.1 *M* phosphate buffer (pH 6.0), (B) 25% methanol–15% THF in 0.1 *M* phosphate buffer (pH 6.0), (C) 40% methanol in water. Isocratic elution with A for 24 min, a linear gradient of 100% A to 100% B over 30 min, isocratic elution with B for 6 min and isocratic elution with C for 12 min were performed successively. Fig. 5 shows that all the derivatives are eluted with reasonably good separation except NBD–Ile (peak p) and NBD–Leu (peak q). The average deviation of retention times for the eighteen amino acid derivatives was 1.35% ($n = 5$), the largest being 5.37% (for Asp) and the lowest 0.19% (for Leu). This deviation might be caused by evaporation of THF at room temperature (*ca.* 25°C). Fig. 5 shows that NBD–ε-aminocaproic acid (peak m) was a good internal standard. A linear relationship was obtained for the peak-height ratio of NBD–ε-aminocaproic acid from 0.5 to 100 pmol for each NBD–amino acid. The average deviation of 10 pmol of each amino acid was 2.78% ($n = 5$); the largest was 5.60% for NBD–Pro and the lowest was 0.57% for NBD–Glu. The detection limits (signal-to-noise ratio 2) for each amino acid were over a range of 10 fmol except for His, Pro and Met, for which they were *ca.* 100 fmol.

Application to protein hydrolysates

The protein hydrolysates of rabbit pyruvate kinase-M_1, rabbit aldolase A and papain were examined by this method. Fig. 6a–c illustrate the amino acid profiles of each protein hydrolysate. Table I summarizes the results of the intercalibration in terms of ratios of amino acid residues, with the amount of Ala fixed as 1.00. The

composition ratios were almost the same as those in the literature[15–17] except those for Pro which were lower.

DISCUSSION

According to Ahnoff *et al.*[6] a reaction time of 3 min at pH 9.5 and 60°C is required for derivatization of Hyp with NBD-Cl. A longer reaction time may be required to derivatize other amino acids, especially amino acids having primary amino groups since the two amino groups of Lys partially reacted to give two peaks[6] presumably of mono- and bis-derivatives. As reported previously[8], NBD-F reacts faster than NBD-Cl with amino acids. Thus, a reaction time of 1 min at pH 8.0 and 60°C is enough for derivatization of the eighteen amino acids, the presumed components of the protein hydrolysates, plus ε-aminocaproic acid to give one peak for each amino acid as shown in Fig. 5.

Contrary to the fact that OPA derivatives of amino acids, especially Lys and Gly, are less stable and for HPLC must be injected at exactly the same time after reaction[12]. Our data (unpublished) that the NBD derivatives are stable for 2 days when stored in the absence of light in a refrigerator might be advantageous for the analysis of amino acids. The third organic modifier in the eluting solvent, THF, suggested for the efficient elution of ethereal compounds[11], is also favourable for the efficient separation of all the NBD derivatives of the amino acids tested except for NBD–Tyr. The reason why NBD–Tyr is difficult to be eluted by the solvent containing salt is not clear. However, in our preliminary results, NBD–Tyr is converted to a N,O-bis-NBD derivative. It might be a reason for strong affinity of NBD–Tyr to the gel.

Since the effect of ion concentration on the elution of NBD–Arg is not great, the guanidino group in the molecule may not be derivatized. The same phenomena were observed as suggested for OPA–[13] and PTH–Arg[14]. The relatively poorer reproducibility of the retention time of NBD–Asp may be ascribed to the volatility of THF present in the eluting solvent in small amounts. Therefore, the eluting solvents should be prepared freshly before use.

The sensitivity of the present NBD method for amino acids is almost the same as for the OPA method[12], excluding Pro and Hyp to which OPA is not reactive, and might be advantageous for NBD-F against OPA.

Since the NBD-OH peak interferes with the baseline separation of NBD–Gly and NBD–Arg, the final dilution ratio in the reaction solution with 0.005 *M* hydrochloric acid cannot be reduced in our present experiment. However, the intensity of the NBD-OH peak could be lowered by changing the pH of the buffer in the eluting solvent to *ca.* 1 (ref. 8). Thus, under such conditions the dilution ratio with hydrochloric acid may be lowered and a greater quantity of sample can be injected without loss of separation.

When the proposed method was applied to the protein hydrolysates, the amounts of Pro gave a lower value compared to that in the literature[15–17] (Table I) for reasons unknown. However, in a trial experiment the values for the other amino acids are in reasonably good agreement with those in the literature[15–17]. Our results suggest that *ca.* 1–1.5 μg of protein hydrolysates in 10 μl of final sample solution prior to derivatization are appropriate for one experiment. It means, occasionally, even a protein band on a polyacrylamide gel could be supplied for the study of the amino

acid composition. The difficulty which might be encountered is how to avoid interference between the amino acids, ascribed to the solvents used for extraction from the gel and hydrolysis of the protein. We are currently investigating this matter.

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