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Determination of free N-acetylamino acids in biological samples and N-terminal acetylamino acids of proteins

Yasuhiko Kawakami, Takahiro Ohga, Chiemi Shimamoto, Noriko Satoh and Shinji Ohmori*

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-Naka-1, Okayama 700 (Japan)

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ABSTRACT

N-Acetylamino acids were derivatized with 9-anthryldiazomethane to the corresponding esters. The anthryl esters were separated by high-performance liquid chromatography and detected fluorimetrically (excitation at 365 nm; fluorescence emission measured at 412 nm). N-Acetyl derivatives of Asn, Gln, Ser, Thr, Gly, Ala, Tyr, Pro, Met, Val, Ile and Leu as well as N-formyl-Met could be separated and identified in the same chromatographic run. The detection limit was from 0.10 pmol for AcGln to 5.5 pmol for AcIle and AcLeu. When the acetylamino acids listed above were added to the 700 *g* supernatant of a rat liver homogenate, the mean recovery was 72%. AcAla and AcTyr were found in free form in baker's yeast. Proteins with an acetylated N-terminus were digested by a protease, and the peptides formed were treated with an N-acylamino acid-releasing enzyme. This method was applied to end-group determination of four proteins (each 0.5 nmol).

INTRODUCTION

The N-terminal amino acids of proteins can be determined by reacting the protein with 1-fluoro-2,4-dinitrobenzene, dansyl chloride and phenylisothiocyanate, which is currently the most frequently used. Even though amino acid sequences can be determined from as little as 10 pmol of protein, an N-acetylamino acid residue at the N-terminus of a protein cannot be easily identified because of their lack of reactivity with these reagents. Brown and Roberts [1] have reported that approximately 80% of the soluble proteins from Ehrich ascites cells have an N-acetylamino acid at their N-terminal residues, and thus a large percentage of eukaryotic proteins may be acetylated at their N-terminal residues [1,2].

Therefore, many people engaged in protein sequencing have been interested in a method for determining the acetylated N-terminal amino acid of proteins.

Wold [3] proposed a pathway for the N-terminal processing: the methionine residue at the N-terminus of a nascent protein is acetylated with acetyl-CoA and then the N-acetylamino acid residue is subjected to enzymic hydrolysis. The same mechanism is repeated until the mature protein is obtained.

We have studied this mechanism, especially the hydrolase causing the release of N-acetylmethionine, by using rabbit skeletal muscle aldolase as the substrate. In order to study this mechanism, we had to develop a method of identifying N-acetyl amino acids. In this paper the determination of free N-acetylamino acids in biological samples and its application as a method for determining the N-terminal acetylamino acid residue of proteins are reported.

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EXPERIMENTAL

Chemicals

9-Anthryldiazomethane (ADAM) was purchased from Funakoshi (Tokyo, Japan). Sulphopropyl-Sephadex C-25 (SP-Sephadex) was purchased from Pharmacia-LKB (Uppsala, Sweden). The N-acetyl derivatives of L-Pro, L-Asp, L-Gln, L-Asn, L-Lys, L-Arg, L-Tyr, D,L-Glu and D,L-Ser were obtained from Sigma (St. Louis, MO, USA). The N-acetyl derivatives of L-His, Gly, D,L-Leu, D,L-Trp, D,L-Ala and D,L-Met and N-formyl-Met were from Tokyokasei Kogyo (Tokyo, Japan). Ac-L-Thr, Ac-L-Val, Ac-L-Ile, and Ac-L-Phe were kindly supplied by Ajinomoto (Tokyo, Japan). Ac-L-Cys, silica gel K-922 (200 mesh) and other reagents were purchased from Katayama Chemical (Osaka, Japan). Acetonitrile from Wako Pure Chemical Industries (Osaka, Japan) was used after distillation.

Trypsin from bovine pancreas (type III), cytochrome c (Cyt, c) from horse heart (type III) and ferritin from horse spleen (type I) were purchased from Sigma. Lactate dehydrogenase (LDH) from pig heart was from Oriental Yeast (Tokyo, Japan). Acylamino acid-releasing enzyme (AARE) was obtained from Takara Syuzo (Kyoto, Japan). AARE was used after dialysis against 5 m*M* sodium phosphate (pH 7.2), 1 m*M* dithiothreitol (DTT). Purification of the light chain from ferritin and Ca^{2+}-ATPase from rabbit skeletal muscle was carried out according to the methods of Ohhashi *et al.* [4]. Briefly, the procedure was as follows. The protein sample was applied to a 2-cm-wide well of a sodium dodecyl sulphate polyacrylamide gel (dimensions: 75 × 60 × 1.5 mm). After electrophoresis for 3 h at 4°C and 150 V, the protein in the gel was visualized by incubating the gel in 4 *M* sodium acetate. The band containing the desired protein was cut out and fixed in 40% methanol. Protein was eluted electrophoretically into a dialysis tube for 3 h at 4°C and 200 V. After the eluate was concentrated in a Centricon (Amicon Grace, Danvers, MA, USA), the protein was used for the determination of N-acetyl termini. The amount of protein isolated from the 2-cm well in one electrophoretic run was enough to analyse the N-acetyl termini.

Instrumentation

Anthryl esters of N-acetylamino acids were measured by a Shimadzu Model LC-6A liquid chromatograph to which a 250 mm × 4.0 mm I.D. column filled with HiberLiChroCART Supersphere 60 RP-8 (Kanto Chemical, Tokyo, Japan) and a Shimadzu RF-535 fluorescence detector were attached. The fluorescence detector was set to an excitation wavelength of 365 nm and an emission wavelength of 412 nm. The mobile phase was 10 m*M* sodium acetate buffer (pH 6.0)–acetonitrile (64:36, v/v). The flow-rate was 1.0 ml/min and column temperature was 60°C.

Derivatization of N-acetylamino acids by ADAM and preparation of calibration curves

The principle of the method for assaying N-acetylamino acids is shown in Fig. 1. Methanol solutions containing various amounts of N-acetyl derivatives of Asn, Gln, Ser, Thr, Gly, Ala, Tyr, Pro, Met, Val, Ile, Leu and N-formyl-Met were placed in a 5-ml vial and evaporated *in vacuo* at room temperature using a Savant Speed Vac concentrator (Model SVC-100 H, New York, NY, USA). The residue was dissolved in 200 μl of methanol. An equal volume of a 0.1% (w/v) methanolic solution of ADAM, which was prepared just before use, was added to the solution of N-acetylamino acids. The vial was capped

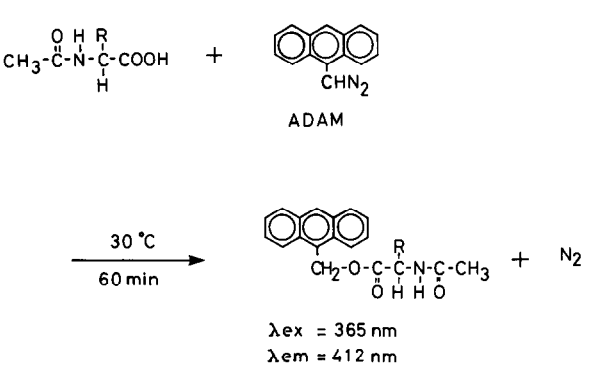

Fig. 1. Derivatization reaction of N-acetylamino acid with ADAM.

tightly and shaken for 60 min at 30°C in the dark. An aliquot of 10–20 μl of the reaction mixture was injected onto the HPLC column.

Preparation of homogenate of baker's yeast and rat liver

Baker's yeast (50 g) (Oriental Yeast) was suspended in 100 ml of cold water. The suspension was homogenized at 4°C for 3 min with 50 g of glass beads of 0.3 mm diameter (Edmund Bahler, Tübingen, Germany). The homogenate was centrifuged at 10 000 *g* for 20 min and the supernatant was used for analysis.

After decapitation of Wistar strain albino rats (male, 200 g), the liver was removed immediately, washed in physiological saline and homogenized in nine volumes of cold water using a Potter–Elvehjem homogenizer with a PTFE pestle. After the homogenate was centrifuged at 700 *g* for 10 min, the supernatant was filtered through a cotton plug in a funnel.

The supernatant of the rat liver (200 μl) or baker's yeast (50 μl) homogenate was placed in a 1.5-ml microtube and four volumes of methanol were added. The mixture was vigorously stirred and centrifuged at 10 000 *g* for 5 min. The supernatant was decanted into another microtube, and the precipitate was sonicated using a water bath-type sonicator for 5 min in 80 μl of methanol. The suspension of the precipitate was centrifuged at 10 000 *g* for 1 min; this washing procedure was repeated three times. All supernatants were combined and allowed to stand at −20°C for 2 h, and the precipitate formed was removed after centrifugation at 10 000 *g* for 5 min.

Determination of free N-acetylamino acids in biological samples

The deproteinized solutions from the homogenates of baker's yeast and rat liver were evaporated to dryness in a Savant Speed Vac concentrator. Methanol (300 μl) was added to the residue and the mixture was sonicated for 5 min. To the suspension, 100 mg of silica gel were added and mixed well. The suspension was centrifuged at 10 000 *g* for 5 min, and 100 μl of the supernatant were discarded. To the precipitate 100 μl of methanol were added and the suspension was vigorously shaken, followed by sonication and centrifugation as described above. The supernatant (100 μl) was pipetted into a 1.5-ml microtube and derivatized with ADAM. A 10-μl aliquot was analysed by HPLC.

Method of determining the N-terminal N-acetylamino acid of proteins and its limit of sensitivity

An aliquot (0.5–25 μl) from a solution containing 2 mg of protein per ml was transferred to a 0.5-ml microtube and heated at 100°C for 3 min. After cooling, one third the volume of 400 m*M* ammonium bicarbonate was added and a 1 mg/ml solution of trypsin was added so that the trypsin/sample protein ratio was 1:50 (w/w). The mixture was incubated at 37°C for 4 h, after which the same amount of trypsin was again added to the mixture and the incubation continued for a further 12 h. The incubation mixture was heated at 100°C for 3 min to terminate the digestion and then dried under vacuum in a Savant Speed Vac concentrator. To the residue 25 μl of 10 m*M* sodium phosphate (pH 7.2) and 0.1 m*M* DTT were added and the mixture was sonicated to suspend the residue. AARE, which releases an N-acetylamino acid from a peptide having an N-acetylamino acid terminus, was added (0.025 U per nmol of protein) to the suspension and allowed to react for 16 h at 37°C. The mixture was heated at 100°C for 3 min and concentrated under reduced pressure. The residue was dissolved in 0.1 ml of 1 m*M* formic acid applied onto an SP-Sephadex column (10 mm × 7 mm I.D.), which was previously equilibrated with 1 m*M* formic acid. The column was eluted with 1.2 ml of 1 m*M* formic acid. The eluate was transferred into a 1.5-ml microtube, frozen at −20°C and dried under reduced pressure as mentioned above. A 30-μl aliquot of methanol and 10 mg of silica gel were added to the tube, which was then centrifuged at 10 000 *g* for 5 min. An aliquot of 12.5 μl from the supernatant was placed into a 0.5-ml microtube and N-acetylamino acids were derivatized as described under *Derivatization of N-acetylamino acids*.

Protein determination

Protein determinations were performed using the method of Lowry *et al.* [5] and the Biuret method [6].

RESULTS

Chromatographic separation

Each N-acetylamino acid (0.5 nmol for AcAsn, AcGln, AcSer, AcThr, AcGly, AcAla and AcTyr and 1.0 nmol for AcPro, AcMet, AcVal, AcIle and AcLeu) and N-formyl-Met (1.0 nmol) were derivatized with ADAM and the esters were analysed by HPLC. As shown in Fig. 2b, AcAsn, AcGln, AcSer, AcThr, AcGly, AcAla, AcTyr, AcPro, AcMet, AcVal, AcIle, AcLeu and N-formyl-Met could be analysed. However, AcCys, AcArg, AcLys, AcHis, AcPhe and AcTrp did not yield identifiable peaks under these conditions.

The peaks of AcAsp and AcGlu overlapped. Although many peaks arose form ADAM itself (see Fig. 2a), they were eluted between the AcVal and AcIle peaks and thus did not interfere with the measurement of N-acetylamino acids described above.

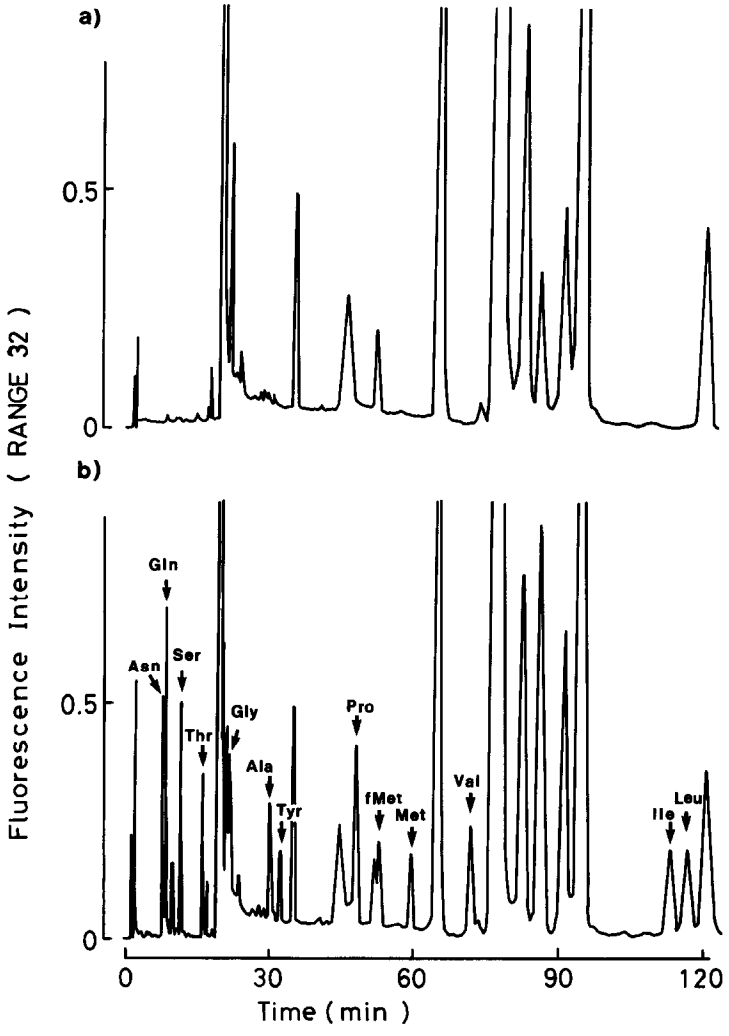

Fig. 2. High-performance liquid chromatograms of anthryl esters derived from authentic N-acetylamino acid. A methanolic solution of twelve N-acetylamino acids and N-formyl-Met was esterified by ADAM at 30°C for 60 min (b). (a) Chromatogram of a methanolic solution of ADAM after incubation at 30°C for 60 min.

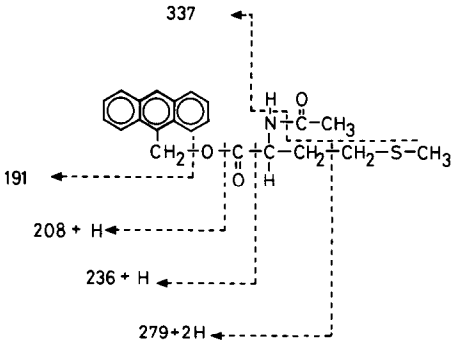

Fig. 3. The anthryl ester of AcMet. Mass spectrometry values are shown.

Mass spectrometry (MS)

In order to identify each peak, corresponding peak fractions from several chromatographic runs were combined, concentrated and rechromatographed on the HPLC column. MS for the anthryl ester of AcMet was successful, giving *m/z* 381 (78.2), 337 (17.3) 279 (9.1), 236 (12.7), 208 (98.2) and 191 (100%). Values of *m/z* less than 191 are not cited here. The molecular ion peak was at *m/z* 381 and other observed peaks were assigned as illustrated in Fig. 3. However, MS of the other esters was unsuccessful because they could not be purified by rechromatography owing to their instability.

Calibration curves and detection limits of free N-acetylamino acids

Various amounts of N-acetylamino acids and N-formyl-Met were mixed and then reacted with ADAM and analysed by HPLC. The relationships between fluorescence intensity and the amount (pmol) of anthryl ester, coefficients of correlation and detection limits are summarized in Table I.

Recovery test of free N-acetylamino acids and N-formyl-Met

Various amounts of N-acetylamino acids and N-formyl-Met were added to 200 μl of rat liver 700 *g* supernatant and the recoveries determined as described under *Determination of free N-acetylamino acids in biological samples*. The average

TABLE I

RELATIONSHIPS OF CALIBRATION CURVES OF N-ACETYLAMINO ACIDS BY HPLC

N-Acetyl-amino acid	Range on the fluorescence detector	$y = ax + b$		r	n	Detection limit (pmol)	
		a	b			Lower	Upper
N-AcAsn	16	28.9	+0.113	0.993	5	0.121	0.603
	32	9.01	+1.22	0.922	9	0.603	12.1
N-AcGln	16	39.4	−0.161	0.994	5	0.101	0.507
	32	11.9	+0.159	0.999	10	0.253	15.2
N-AcSer	16	28.6	−0.515	0.992	5	0.131	0.656
	32	9.89	+1.17	0.999	9	0.328	19.7
N-AcThr	16	14.3	−2.96	0.959	3	0.549	2.20
	32	8.10	−2.92	0.999	6	1.37	16.5
N-AcGly	32	2.08	+3.36	0.997	8	4.25	51.0
N-AcAla	16	16.7	+1.30	0.983	4	0.239	0.798
	32	7.00	−5.41	1.00	7	0.798	31.9
N-AcTyr	32	7.98	−2.99	0.992	6	0.878	8.78
N-AcPro	32	2.60	+5.39	0.998	8	3.02	42.3
N-AcMet	32	2.46	+0.403	0.994	7	1.26	20.1
N-AcVal	32	4.26	+0.387	0.997	11	0.335	50.2
N-AcIle	32	0.593	+1.49	0.997	7	5.46	54.6
N-AcLeu	32	0.656	+1.29	0.995	7	5.46	54.6
N-fMet	32	2.02	+2.62	0.995	8	3.14	62.7

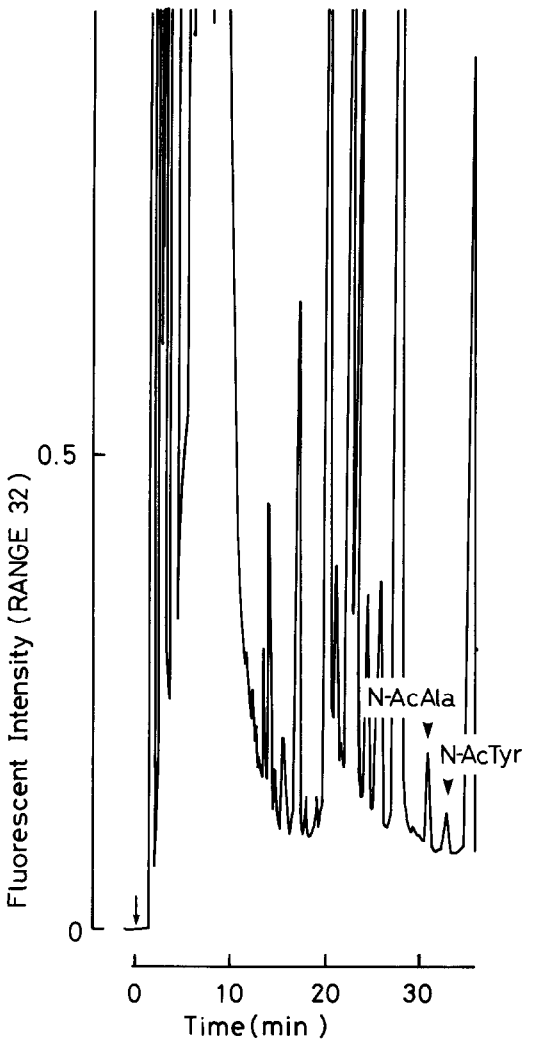

Fig. 4. AcAla and AcTyr in the supernatant of a homogenate of baker's yeast.

recovery of ten N-acetylamino acids was 72.4 ± 13%. However, AcGly and AcLeu could not be determined, because of interference from substances intrinsic to rat liver. No naturally occurring free N-acetylamino acids were found in the supernatant of rat liver homogenate by this method.

Free N-acetylamino acids in homogenate of baker's yeast

As shown in Fig. 4 free AcAla and AcTyr were found in the 10 000 *g* supernatant of baker's yeast homogenate. Their contents were 1.14 ± 0.089 and 1.46 ± 0.039 nmol/mg of protein, respectively.

Determination of N-acetylamino acid residues at the N-termini of proteins and their detection limit

Ferritin light chain, Cyt c, LDH and Ca^{2+}-ATPase were chosen as standard proteins since they are known to have an N-acetylamino acid residue at their N-terminus and they are commercially available.

In Table II, the recoveries of N-terminal residues from the four standard proteins are listed. When 0.5 nmol of protein were used for this procedure, a well defined and distinct peak was obtained upon HPLC. In order to determine the detection limit of the N-acetylamino acid at the N-terminus, various amounts of ferritin light chain were subjected to the labelling procedure; the resultant AcSer was analysed by HPLC. The results are shown in Table III; even 50 pmol of protein could be analysed with good yield.

TABLE II

DETERMINATION OF N-ACETYLAMINO ACIDS AT THE TERMINI OF PROTEINS

A 0.5-nmol aliquot of each protein was digested by trypsin and AARE, and the N-acetylamino acids released were derivatized by ADAM. Anthryl ester was analysed by HPLC.

Sample protein	N-Terminal residue	Determined N-acetyl-amino acid (pmol)	Yield (%)
Ferritin light chain	N-AcSer	353	70.6
Cyt c	N-AcGly	208	41.1
LDH	N-AcAla	150	30.0
Ca^{2+}-ATPase	N-AcMet	221	44.2

TABLE III

DETECTION LIMIT OF N-ACETYLSERINE RESIDUE AT THE N-TERMINUS OF FERRITIN LIGHT CHAIN

Various amounts of ferritin light chain were digested with trypsin and AARE, and the AcSer was labelled with ADAM. The anthryl ester of AcSer was determined by HPLC.

Amount of sample (pmol)	Peak height at range 16 (mm)	Determined N-AcSer (pmol)	Recovery (%)
0	0	0	–
50	19	28.0	56.0
75	35	56.8	75.7
100	29	46.0	46.0
200	86	149	74.5

DISCUSSION

Since N-acetylamino acids cannot be effectively extracted from aqueous solution by any organic solvent and have no reactive group other than a carboxyl group, they had to be derivatized to the ester or amide in aqueous medium or methanol.

In order to identify very small amounts of N-acetylamino acids, the amino acid should be labelled by reagents which fluoresce or contain a halogen group. Very sensitive detection of fluorescent derivatives can be achieved by HPLC coupled to a fluorescence detector, whereas that of halogenated derivatives is achieved by gas chromatography and electron-capture detection. We have unsuccessfully tried to label N-acetylamino acids with many reagents, such as 9-aminomethylphenanthrene, 9-chloromethylanthracene, 9-aminomethylanthracene, 4-bromomethyl-7-methoxycoumarin and pentafluorobenzylchloride. In all cases, the derivatization reactions were not quantitative. For these reasons we chose ADAM as the labelling agent [7]. However, the purchased chemical was not very pure. Many efforts to obtain pure ADAM ended in failure: we could not further purify ADAM obtained commercially and failed to synthesize pure ADAM. As shown in Fig. 1, since the free carboxyl group on the amino acid is esterified, the reaction has to proceed under acidic conditions. When the reaction was run in the presence of an inorganic acid such as sulphuric or phosphoric acid, the ester was not obtained in good yields. After many trials, it was found that silica gel produced the best yields. This was very important and without silica gel treatment the determination method would not succeed.

The N-acetylamino acid which could be detected with the highest sensitivity was AcGln; the detection limit was 0.10 pmol. The least sensitively detected amino acids were AcIle and AcLeu, whose limit of detection was 5.5 pmol.

About 80% of the soluble proteins from Ehrlich ascites cells and 90% of the proteins from mouse L-cells are acetylated at the N-termini [1,2]. According to Arfin and Bradshaw [8], 95% of the N-acetylamino acids in eukaryotic proteins are AcSer, AcAla, AcMet, AcGly and AcThr. Therefore, the method presented here could be used to determine the acetylated termini of most eukaryotic proteins.

In 1982 Tsunasawa and Narita [9] developed an identification method for acetylamino acid at the amino termini in proteins. However, this method requires 10–100 nmol of protein and seven steps, including two steps for digestion and five steps for column chromatography. Recently Wellner *et al.* [10] presented a method by which AcSer and AcThr as the N-terminal residues of proteins could be determined. Their method is based on the fact that when proteins with AcSer or AcThr at the N-terminus are treated with anhydrous trifluoroacetic acid they can be deter-

mined by Edman degradation. The method presented here is better than all other methods in terms of its range of application and the level of sensitivity. The minimum amount of protein required for end-group determination was 50 pmol.

When the method presented here was applied to the supernatant of a homogenate of rat liver, no acetylated amino acid could be found in it. However, we found AcAla and AcTyr in the supernatant of a homogenate of baker's yeast. Rothenbuehler *et al.* [11] isolated and identified AcAla and AcTyr from baker's yeast. The physiological significance of acetylated amino acids in yeast is still uncertain.

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