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# *N*-ACETYLASPARTIC ACID IN G-ACTIN

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SUMMARY

A ninhydrin-negative peptide fraction obtained from pronase digestion of G-actin was isolated on a Dowex-50 X-2 ($H^+$) column. Analysis of this fraction by gas chromatography showed that most of the acetyl content of the original actin was recovered. The presence of an acetyl group in the peptide fraction was also demonstrated by hydrazinolysis. This fraction consists mainly of a tripeptide which, upon degradation with carboxypeptidase A (EC 3.4.2.1), had an amino acid sequence of *N*-acetyl–Asp–Glu–Thr. It is suggested that *N*-acetylaspartic acid is the $NH_2$-terminal residue of G-actin.

## INTRODUCTION

The absence of a free amino terminal residue in G-actin (mol. wt. about 60000 (ref. 1)) was demonstrated by LOCKER[2] some years ago and confirmed more recently by KRANS *et al.*[1]. Because of the increasing number of well-characterized proteins found in recent years to be N-acetylated at their amino terminal[3–12], the possibility that actin might also be acylated was investigated. The results of this investigation will show that actin is, indeed, an N-acetylated protein. Thus when G-actin was hydrolyzed to small peptides with pronase, a ninhydrin-negative peptide fraction containing acetyl groups was isolated from the digest. Analysis of this fraction then led to the identification of a tripeptide with the sequence *N*-acetyl–Asp–Glu–Thr.

## EXPERIMENTAL

### *Materials*

Aspartylglutamic acid was prepared according to the procedure of BRYANT *et al.*[14].

*N*-acetyl-α-aspartylglutamic acid: To 100 mg of aspartylglutamic acid in 2.5 ml of water, 0.1 ml of acetic anhydride was added gradually and with stirring while maintaining the pH at 6–7 with 1 N NaOH. The reaction mixture was then applied to a 0.9 cm × 10 cm column of Dowex-50 X-2 ($H^+$) resin, and the column washed with water. The first 50 ml were collected and flash-evaporated to dryness. The residue was dissolved in 0.5 ml of acetone and the N-acetylated dipeptide was precipitated by adding 6 ml of ether. This procedure was repeated 3 times, and a white semi-crystalline residue was obtained which melted at room temperature after drying *in*

*vacuo*. Analysis* calculated for *N*-acetyl-α-aspartylglutamic acid: C, 43.42; H, 5.30; N, 9.21 %. Found: C, 43.25; H, 5.40; N, 9.31 %.

*N*-acetylglutamic acid and *N*-acetylaspartic acid were obtained from Mann Research Laboratories.

*Amino acid analyses*

In general, 2 mg of protein, or 1.0 μmole of peptide was hydrolyzed with 3.0 ml of 6 N HCl in evacuated and sealed Pyrex tubes (20 mm × 150 mm) at 110° for 24 h and 48 h. The hydrolyzates were flash-evaporated to dryness and dissolved in 0.2 N citrate buffer (pH 2.8). Amino acid analyses were performed in duplicate on a Technicon automatic analyzer.

G-actin was prepared from the acetone powder[15] according to the procedure of CARSTEN AND MOMMAERTS[16] except that the polymerization step was carried out twice. The final pellet was homogenized in distilled water at pH 7.5 and the actin solution was treated with Dowex-1 X-8 ($Cl^-$) resin (50 mg/ml) for 10 min to remove bound nucleotides and filtered through a coarse sintered glass funnel. The exact protein concentration was determined from its total amino acid content. The amino acid composition of our preparation was in agreement with that reported recently for highly purified actin[1,16].

*Digestion with pronase*: In a typical experiment, 600 mg of G-actin (in 200 ml) from the above preparation was denatured by heating at 90° for 2 min. After cooling, the solution was incubated with 6 mg of pronase (*Streptomyces griseus* protease, Grade B, Lot No. 502117) at pH 7.5 and 23°; the pH was maintained constant by addition of 1.0 N NaOH with the aid of a Polarad automatic titrator. The digestion was stopped by lowering the pH to 4.0 with 6 N HCl. A small precipitate appeared which was spun down by centrifugation at 9000 rev./min for 15 min. The supernatant was applied to a 2.3 cm × 16 cm column of Dowex-50 X-2 ($H^+$) and the column washed with water. The ninhydrin-negative fraction was collected in a volume of 700 ml, the solution lyophilized to dryness, and the residue dissolved in 10 ml of water. This solution will be referred to as the "unretarded Dowex-50 actin fraction." An identical experiment was performed on 6 mg of pronase in the absence of actin. The residue obtained after chromatography on Dowex-50 and lyophilization was also dissolved in 10 ml of water. This solution will be referred to as the "pronase control."

*Digestion with carboxypeptidase A*: Carboxypeptidase A (EC 3.4.2.1) was a 3-times crystallized suspension treated with DFP (Worthington Biochemical Corporation Lot No. 6131). Before use, the suspension was centrifuged and the precipitate washed 3 times with 10 volumes of water. The carboxypeptidase pellet was dissolved in 10 % LiCl at a concentration of 20–30 mg/ml. Enzymic reaction was initiated by the addition of substrate solution buffered at the appropriate pH and continued by stirring at 23°; the final LiCl concentration was 1–2 % and the carboxypeptidase concentration, 2–3 mg/ml. The reaction was stopped by lowering the pH to about 2.0 with 6 N HCl, and the precipitated protein separated by centrifugation. The supernatant was applied directly to a Technicon automatic amino acid analyzer for quantitative estimation of the amino acids released. These values in turn were corrected by subtracting "carboxypeptidase blanks" run in the absence of substrate. These corrections, however, were negligible.

* Microanalyses by Schwarzkoff Microanalytical Laboratory, Woodside 77, N.Y.

*Determination of acetic acid by gas-phase chromatography*: A freshly prepared solution of G-actin (150 mg) was made 0.01 N in HCl and lyophilized for 48 h to remove adventitious acetic acid. The dry protein was transferred to a Pyrex tube, 25 mm × 150 mm, and 4.0 ml of $H_2SO_4$ (Mallinckrodt analytical reagent) were added. The tube was evacuated, sealed, and heated at 100° for 24 h. In the case of the "unretarded Dowex-50 actin fraction", a 3.0-ml sample derived from 150 mg of actin was flash-evaporated 3 times in a Pyrex tube, 20 mm × 150 mm, at 30° in the presence of 0.01 N HCl. The residue was hydrolyzed in 2.0 ml of $H_2SO_4$ as described before. Two additional samples were prepared and carried through the entire procedure: (1) a "reagent blank" consisting of 2.0 ml of $H_2SO_4$, and (2) a blank consisting of 3.0 ml of "pronase control" solution. The hydrolyzates were chilled, transferred to the distillation-diffusion apparatus of BARTLEY[17], and the distillation procedure was carried out. The chilled distillates were transferred to 40-ml conical centrifuge tubes, and propionic acid (1.92 μmoles in 1.0 ml of water) was added as an internal standard. The solutions were made alkaline with 1 N NaOH and flash-evaporated to dryness at 40°. The residues were dissolved in 0.1 ml of 0.2 M $H_3PO_4$ and extracted 3 times with 0.5 ml of anhydrous diethyl ether; after each extraction the tubes were centrifuged and the ether layer transferred to small test tubes. The combined ether extract was evaporated to one-fifth of its original volume in a stream of $N_2$ for 2 min at about 10°. Aliquots of this solution were chromatographed on a F and M Model 1609 flame ionization electrometer and detector. The column was a 6 ft × 0.375 in Cu coil of 3 % polyglycol succinate ester on chromosorb W. The injection port and the detector were maintained at 125°. The He flow rate was 30 ml/min. The analyses were conducted by injecting 8 μl of the test samples (ethyl ether solutions) into the column equilibrated at 35°. After 10–12 min the column temperature was programmed at a rate of 2.9°/min to elute acetic acid (62–64°) and propionic acid (71–73°). Quantitation of the two components was done by proportioning the peak heights of test samples with the peak heights of standard acetic and propionic acid solutions in ethyl ether*. Each determination was carried out in duplicate and the agreement was within 15 %.

## RESULTS AND DISCUSSION

Two main steps are involved in the isolation of an N-acetyl peptide from a protein: (1) the hydrolysis of the protein chain to small fragments without hydrolyzing the N-acetyl bond, and (2) the separation of the acylated peptide from a large quantity of non-acylated peptides. Usually the protein is digested with an endopeptidase and the digest washed with water on a column of Dowex-50 X-2 ($H^+$) resin[3]. The resin firmly binds the peptides with free amino groups and allows N-acetylated and pyroglutamyl peptides to pass through the resin unretarded. This simple procedure will fail, however, when the N-acetylated residue is a basic amino acid or when the peptide contains a basic amino acid.

In the present work, several preparations of G-actin were digested with pronase for periods of 1 h and 20 h at pH 7.5 and 23°. Pronase digestion for 1 h was sufficient to release from actin a peptide fraction which was unretarded on the Dowex-50 column. About one-fourth of the total peptide bonds of actin was hydrolyzed by pronase in 1 h as indicated by the uptake of NaOH. The 20-h pronase treatment was

* Gas chromatography was performed by the Analytica Corporation, New York 16, N.Y.

performed only occasionally to minimize the formation of pyroglutamic acid and pyroglutamyl peptides which add to the problems of purification.

Table I shows the acetyl content of G-actin, and of the "unretarded Dowex-50 actin fraction" obtained by gas chromatography. Acetyl groups are found in actin and in the "unretarded Dowex-50 actin fraction", but not in the reagents or in the pronase itself. No additional peaks aside from the added propionic acid could be found on the chromatogram, and in view of the ability of gas chromatography to resolve completely the lower fatty acids there is evidence that actin contains acetyl groups. The table shows, furthermore, that most of the acetyl content of actin is recovered in the "unretarded Dowex-50 actin fraction". The high recovery of acetyl groups indicates that only a small fraction of them could have been attached to a basic peptide or to a basic amino acid and therefore withheld by the Dowex-50 column.

Table II shows the amino acid analysis of the "unretarded Dowex-50 actin fraction" obtained from three separate preparations of G-actin digested for 1 h, and from one preparation digested for 20 h. (In a control experiment, no amino acid

TABLE I

ACETYL CONTENT OF G-ACTIN

The acetyl content of the different samples was determined by gas chromatography as described under EXPERIMENTAL. The "unretarded Dowex-50 actin fraction" was obtained after 1 h of pronase treatment. The amino acid composition of the "unretarded Dowex-50 actin fraction" is shown in Columns 3a and 3b of Table II.

| *Sample* | *Moles acetyl groups per 60 000 g actin* |
|---|---|
| G-actin | 1.7 |
| "Unretarded Dowex-50 actin fraction" | 1.3 |
| "Reagent blank" | < 0.1 |
| "Pronase control" | < 0.1 |

TABLE II

AMINO ACID COMPOSITION OF THE "UNRETARDED DOWEX-50 ACTIN FRACTION"

In Columns 1, 2, 3, and 4, the analysis from four different actin preparations is shown. In Columns 1, 2, 3a, and 4, hydrolyses were performed in 6 N HCl for 24 h. In Column 3b, hydrolysis was with carboxypeptidase A at an enzyme to substrate ratio of 1:5 for 12 h at pH 7.0, 23°. The reaction mixture contained in a volume of 1.0 ml: 0.25 μmole of peptide (based upon glutamic acid), 1.75 mg carboxypeptidase, 0.1 mmole Tris, and 2% (w/v) of LiCl. Columns 3a and 3b compare the analyses of the same "unretarded Dowex-50 actin fraction."

| *Amino acid* | *Moles per 60 000 g actin (digested with pronase for 1 h)* | | | | *Moles per 60 000 g actin (digested with pronase for 20 h)* |
|---|---|---|---|---|---|
| | *1* | *2* | *3a* | *3b* | *4* |
| Aspartic acid | 1.9 | 1.7 | 1.7 | 0.88 | 2.5 |
| Threonine | 1.1 | 0.67 | 0.65 | 1.2 | 0.88 |
| Glutamic acid | 1.8 | 1.4 | 1.5 | 1.2 | 2.7 |
| Alanine | 0.43 | 0.33 | 0.29 | 0.35 | 0.30 |

could be found in the eluate from the Dowex-50 X-2 column when a solution of pronase in the absence of actin was carried through the same procedure used for the digestion.) There is only a small variation in the alanine content of the various fractions, and a much larger variation in the threonine content. The latter may be due to destruction of threonine during HCl hydrolysis. The ratio of aspartic acid to glutamic acid is nearly constant in all the fractions, their amount being higher in the 20-h digest. Since all these fractions contain glutamic acid, a distinction between pyroglutamic acid and glutamic acid was attempted by treating one of the fractions with carboxypeptidase A. Columns 3a and 3b in Table II compare the amino acid analysis of the same fraction after hydrolysis in 6 N HCl for 24 h and after hydrolysis with carboxypeptidase for 12 h at pH 7.0, respectively. It can be seen that most of the glutamic acid is released by carboxypeptidase, which shows clearly that a high percentage of glutamic acid is not present in the form of pyroglutamic acid because carboxypeptidase does not attack the pyroglutamyl ring[18]. A representative sample of the "unretarded Dowex-50 actin fraction" (containing 0.5 $\mu$mole of glutamic acid) produced no color when submitted to the ninhydrin assay according to the method of MOORE AND STEIN[19], and failed to show any peaks on the automatic amino acid analyzer. When chromatographed on paper with two different solvent systems (Table III) only one spot could be revealed. When submitted to hydrazinolysis[20] and when the hydrazinolysate was chromatographed on paper, the presence of acetyl hydrazide was clearly demonstrated (Table III). No formyl or propionyl hydrazides, which possess $R_F$ values different from that of acetyl hydrazide in this solvent system[3], could be detected on the chromatogram. It appears, therefore, that mainly acetyl groups are

TABLE III

PAPER CHROMATOGRAPHY OF THE "UNRETARDED DOWEX-50 ACTIN FRACTION" OBTAINED AFTER DIGESTION WITH PRONASE FOR 1 h

Chromatography was performed on Whatman 3 MM paper. "Unretarded Dowex-50 actin fraction": samples containing 1.0 $\mu$mole of glutamic acid were applied. After chromatography, the papers were allowed to dry at room temperature for 3 days and then dipped in bromcresol green indicator[22]. "Unretarded Dowex-50 actin fraction" after hydrazinolysis: a sample containing 1.0 $\mu$mole of glutamic acid was evaporated to dryness and heated with 1.0 ml of anhydrous hydrazine (about 95%, Eastman Organic Chemicals) at 100° for 10 h in an evacuated, sealed Pyrex tube. The excess hydrazine was removed by flash-evaporation at room temperature, the residue dissolved in water and chromatographed; 0.5 $\mu$mole of acetyl hydrazide was applied as a marker to the same sheet of paper. After drying for a few hours at room temperature, the paper was dipped in the mixture composed of 0.1 N $AgNO_3$–5 N $NH_4OH$–ethanol (1:1:2, v/v).

| *Substance* | *$R_F$ values (descending)* | *Solvent* |
|---|---|---|
| "Unretarded Dowex-50 actin fraction" | 0.16 | Acetic acid–water–butanol (15:25:60, v/v) |
| "Unretarded Dowex-50 actin fraction" | 0.21 | Pyridine–water–butanol (1:1:1, v/v) |
| "Unretarded Dowex-50 actin fraction" after hydrazinolysis | 0.69 | Pyridine–aniline–water (9:1:4, v/v) |
| Acetyl hydrazide | 0.69 | Pyridine–aniline–water (9.1:4, v/v) |

present in the "unretarded Dowex-50 actin fraction". All these observations suggest that this fraction consists mainly of an acetylated tripeptide composed of aspartic acid, threonine, and glutamic acid. In addition, this fraction probably also contains the overlapping tetrapeptide composed of aspartic acid, threonine, glutamic acid, and alanine. No dipeptide or amino acid, however, seem to be present since these compounds have much higher $R_F$ values than the tripeptide (see Fig. 2).

Treatment of the "unretarded Dowex-50 actin fraction" with pronase or pepsin, at a molar ratio of enzyme to substrate as high as 1:5, released only alanine, but failed to split the rest of the peptide or to hydrolyze the N-acetyl bond. Attempts to remove the acetyl group with the enzymes deacylase I and deacylase II (*N*-acyl-amino-acid amido hydrolases) (ref. 21) also failed. More successful, however, were the experiments with carboxypeptidase A, the results of which are shown in Fig. 1. There

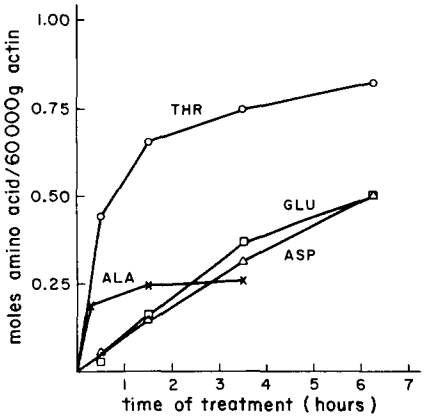


Fig. 1. Treatment of "unretarded Dowex-50 actin fraction" (from a digestion with pronase for 1 h) with carboxypeptidase A. Reaction conditions: enzyme to substrate ratio 1:16, pH 6.8, 28°, 0.1 M Tris, 1% (w/v) of LiCl. See EXPERIMENTAL SECTION for more details.

is an immediate and complete release of alanine. In addition, there is a preferential release of threonine over glutamic acid and aspartic acid which suggests that threonine is at the carboxyl terminal position in the tripeptide. Because glutamic and aspartic acids were released at comparable rates, no conclusion could be drawn concerning their sequential position. Advantage was taken, however, of the wide pH–activity range of carboxypeptidase A to effect differential release of glutamic and aspartic acids. Thus, in contrast to the behavior of carboxypeptidase in the neutral pH range, between pH 5.5 and 6.0 glutamic acid was released at a faster rate than aspartic acid. Since alanine is released completely, it appears that at some intermediate stage of the carboxypeptidase digestion the reaction mixture would contain three N-acetylated components, namely a tripeptide, a dipeptide, and an amino acid. It should be possible to separate and identify these components by paper chromatography. The results of such an experiment are shown in Fig. 2. In Row 3 on the chromatogram, a sample of the "unretarded Dowex-50 actin fraction" after treatment with carboxypeptidase A and after elution from a Dowex-50 X-2 column is compared with known synthetic compounds: *N*-acetylglutamic acid (Row 1), *N*-acetylaspartic acid (Row 2), *N*-acetyl-α-aspartylglutamic acid (Row 4). It may be seen that the actin fraction yields 4 spots,

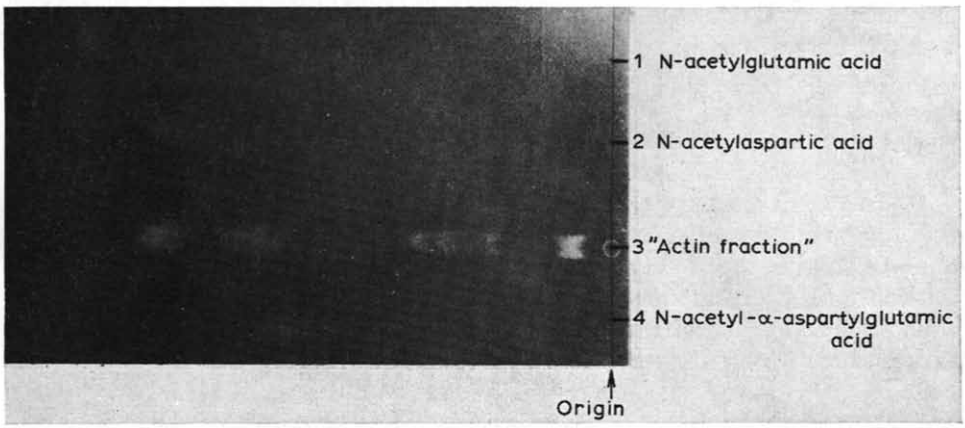


Fig. 2. Chromatography of the "unretarded Dowex-50 actin fraction" after carboxypeptidase-A treatment. The "unretarded Dowex-50 actin fraction" was obtained after digestion with pronase for 1 h. Carboxypeptidase-A treatment was performed at pH 5.7, 25°, for 5.5 h at an enzyme to substrate ratio of 1:5. The reaction mixture contained in a volume of 4.3 ml: 4 μmoles of substrate (based on glutamic acid content), 0.3 M acetate buffer, 1.5% (w/v) of LiCl. The reaction was stopped by lowering the pH to 3.0 with 6 N HCl, and the precipitate separated by centrifugation. The supernatant was applied to a 0.9 cm × 20 cm column of Dowex-50 X-2 ($H^+$), and the column was washed with water. The first 80-ml fraction was flash-evaporated to dryness, and the residue was dissolved in 0.1 ml of water and applied to a sheet of Whatman 3 MM paper (Row 3). Also applied were: 0.5 μmole of *N*-acetylglutamic acid (Row 1); 0.5 μmole of *N*-acetylaspartic acid (Row 2); 0.5 μmole of *N*-acetyl-α-aspartylglutamic acid (Row 4). The chromatogram was developed with the mixture acetic acid–water–butanol (15:25:60, v/v), dried for 3 days at room temperature, and dipped in bromcresol green indicator[22]. The $R_F$ values of the N-acetylated compounds are: *N*-acetylglutamic acid, 0.63; *N*-acetylaspartic acid, 0.54; *N*-acetyl-α-aspartylglutamic acid, 0.43. In Row 3, starting at the origin, 4 spots are described: spot No. 1, $R_F$ 0.05; spot No. 2, a group of 3 closely spaced spots, average $R_F$ 0.20; spot No. 3, $R_F$ 0.43; spot No. 4, $R_F$ 0.54.

one identical with *N*-acetylaspartic acid, another identical with *N*-acetyl-α-aspartylglutamic acid, and two with much lower $R_F$ values. The 4 spots of the "actin fraction" were eluted from the paper, hydrolyzed with 6 N HCl for 24 h, and analyzed for their amino acid content. No amino acids could be found in spot No. 1*; spot No. 2 gave 0.14 μmole of aspartic acid, 0.15 μmole of glutamic acid, and 0.032 μmole of threonine; spot No. 3, 0.46 μmole of aspartic acid, and 0.58 μmole of glutamic acid; spot No. 4, 0.78 μmole of aspartic acid. It can be concluded that the "unretarded Dowex-50 actin fraction" consists mainly of an N-acetylated tripeptide with the sequence *N*-acetyl–Asp–Glu–Thr.

In conclusion, the data presented here suggest that G-actin contains an N-acetylated amino terminal. This conclusion is based on the identification of acetyl groups (1) by gas chromatography, (2) as acetylhydrazide, and (3) by identification of the acetylated amino acid residue as *N*-acetylaspartic acid.

Myosin, which is intimately associated with actin in the muscle cell, has also been reported recently to possess N-acetylated amino terminals[12]. A total of six different N-acetylated amino acids have been identified in naturally occurring proteins[3–13]. The finding of *N*-acetylaspartic acid in actin represents a new addition to this list.

* No further attempts were made to characterize the material in this spot. It will be noted, however, that any acidic compound will yield a yellow spot.

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