Purification and Characterization of an N-Acylaminoacyl-peptide Hydrolase from Rabbit Muscle*

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An N-acylaminoacyl-peptide hydrolase has been purified to homogeneity (7,000-fold with 20% yield) from rabbit muscle. This overall enrichment and its general properties as a soluble protein suggest that it is of cytosolic origin and not a component of ribosomes or other cellular organelles. The enzyme has an M, of 230,000–245,000 and a subunit M, of 76,000–80,000. An extensive survey of the substrate specificity of the pure enzyme reveals that our earlier conclusions (Radhakrishna, G., and Wold, F. (1986) J. Biol. Chem. 261, 9572–9575) that the enzyme is specific for Ac-Met-peptides are wrong. The enzyme catalyzes the rapid removal of Ac-Thr, Ac-Ala, Ac-Met, Ac-Ser, and more slowly Ac-Gly from peptides of different lengths. Other acetylated amino acids (Cys, Tyr, Asp, Val, Phe, Ile, Leu) may be removed at 1% or less of the rate of the above good substrates from some peptide substrates. The nature of the amino acid in the second position of the acetylated peptide generally has only a minor effect on the reaction rate; however, with charged amino acids (Arg, Asp) in the second position the reaction is retarded, and with proline it is virtually abolished. Except for slow rate of hydrolysis of acetylated dipeptides, the hydrolase does not appear to be severely affected by the peptide length in the range studied (from 2 to 11 amino acid residues). The hydrolase also cleaves formylamino acids from formylated peptides. The biological function of the enzyme is not clear.

A good deal of work has been carried out on the characterization of N-acylaminoacyl-peptide hydrolases over the last several years (1–7). The work has been motivated by at least two separate goals: to shed light on the detailed mechanism of NH2-terminal trimming and acetylation in protein biosynthesis and to generate a hydrolase that can be used as a reagent to unblock acetylated proteins for protein sequencing. Muscle tissue represents an interesting source of such hydrolases. Although some muscle proteins now are known to have either an acetylated or a free NH2 terminus corresponding to the second encoded amino acid, exposed after the removal of the initiator methionine. We reported three years ago (8) that a crude enzyme preparation from rabbit muscle has at least be acetylated before it is removed from the nascent polypeptide chain. On further examination of the assays used in earlier work, it became clear that the conclusions regarding the absolute specificity of the enzyme for Ac1-Met-peptides were wrong, and we undertook to re-examine the hydrolase from rabbit muscle. In the original work, a crude enzyme preparation was obtained by salt-extraction of the ribosomal fraction from muscle, in the present study the entire muscle extract was fractionated by ammonium sulfate to give the starting high activity fraction from which a pure hydrolase could be obtained. The purification and properties of this enzyme are reported in this communication.

EXPERIMENTAL PROCEDURES AND RESULTS

DISCUSSION

Substrates—Most of the substrates used in this work are readily available from commercial sources of peptides acetylated with acetic anhydride. We found it convenient to prepare a group of relatively long peptides differing only in the acetylated NH2-terminal amino acid from a common precursor peptide treated in parallel with the hydroxysuccinimide ester of the different N-acetylamino acids desired as NH2 termini. Rather unexpectedly the reaction was associated with extensive racemization of the activated acetylamino acid. In the case of the octapeptide Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu converted to the acetylated nonapeptide with Ac-Met, Ac-Ala, and Ac-Gly, respectively, as the NH2 termini, both the Ac-Met and Ac-Ala products showed a single molecular ion in the mass spectrum but gave two peaks on HPLC. The two Ac-Met diastereoisomers could be separated and shown to contain about 60% of one component which was completely hydrolyzed by the enzyme and 40% of a second component of the same molecular weight, which was inactive as a substrate. Similarly, the Ac-Ala-peptide showed two components (about 60:40) on HPLC; they had very similar retention times and were not separated. The enzyme hydrolyzed 60% of the mixture leaving the second, 40%, component unaltered. These observations along with the fact that the Ac-Gly-peptide gave a single product completely hydrolyzable by the enzyme show that the two acetyl-l-amino acids were converted to acetyl-d-amino acids during the activation, storage, and coupling. Similar racemization reactions have been observed for other carboxyl-activated amino acid and peptide derivatives (9–11).

1 The abbreviations used are: Ac, acetyl; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; FAB, fast atom bombardment; DTT, dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid]; TFA, trifluoroacetic acid.

2 Portions of this paper (including “Experimental Procedures,” “Results,” Figs. 1–6, and Table III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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This side reaction does not appear to cause any serious problem but needs to be considered for quantification of the data.

**Assay**—In the original work (8) an assay was developed which permitted the direct measurement of \(^{14}C\)Ac-amino acid released from the \(^{14}C\)Ac-peptide substrate. Substrate and product were separated by HPLC, and the fractional amount of radioactivity in the early product peak could be related to the total radioactivity as a function of incubation time and enzyme concentration. While this assay worked well for Ac-Met detection, it was subsequently found that some of the less hydrophobic amino acid derivatives could give ambiguous results. The retention of the hydrophobic acetylamino acids was found to be sensitive to the pH and the ionic strength of the sample applied to the HPLC column. If, for example, the buffering capacity of a sample exceeded the trifluoroacetic acid present in the solvent system in the initial sample, free acetate, Ac-Ala, Ac-Gly, and Ac-Ser will each elute as a double peak, one presumably not retained as the carboxylate salt, and the other retarded and eluted in the proper position as the protonated acid. In some cases involving the crude enzyme preparations in the early work (8), the nonretarded peak was the major product peak and was erroneously identified as free acetate, presumably produced by some unknown contaminating activity in the enzyme preparation. By using excess acid in the preparation of the sample for analysis the retention times could be made more reproducible, but the most important improvement in the assay was achieved by adding to each sample to be assayed the appropriate \(^{3}H\)Ac-amino acid as internal marker. Only when the early product peaks showed coincidence of the \(^{3}H\) and \(^{14}C\) peaks was it concluded that the hydrolysis had produced an acetylamino acid product.

The HPLC assay is obviously time-consuming and tedious, and for the assay of a large number of samples during the enzyme purification, the rapid semiquantitative assay based on the paper chromatographic detection of the ninhydrin-positive dipeptide Leu-Gly released by the action of the enzyme on Ac-Met-Leu-Gly provided a simple and convenient alternative.

**The Enzyme**—The enzyme purification is summarized in Table I. The 7,000-fold purification was achieved by relatively few and simple steps and based on the criteria applied (constant specific activity across the symmetrical peak in gel filtration and a single band on gel electrophoresis), we conclude that the final product is a pure enzyme. Based on the individual data under “Results,” the enzyme has a pH optimum of 6.9, an \(M_c\) of 230,000-245,000, and subunit \(M_c\) of 76,000-80,000, and a \(K_m\) (for the tripeptide Ac-Met-Leu-Gly) of about 1 mM (8). When stored at \(-20^\circ C\) in 0.05 mM sodium phosphate buffer, pH 6.9, containing 2 mM MgCl\(_2\) and 1 mM EDTA, the enzyme appears to be stable over several months.

A good deal of effort was invested in establishing the specificity of the hydrolyase. The relative activity observed with a large number of different peptides is compiled in Table II to illustrate three distinct specificity determinants: the nature of the NH\(_2\)-terminal acetylamino acid, the nature of the second amino acid, and the length of the peptide. Comparing groups of acetylated peptides differing only in the nature of the NH\(_2\)-terminal amino acid, suggests that the enzyme is specific for the five amino acids most commonly found acetylated in eukaryotic proteins in the following order of preference: Thr > Ala > Met > Ser > Gly. The second amino acid does not appear to affect the rate of hydrolysis of the acetylaminoacyl-peptide bond in terms of the bulkiness or hydrophobicity of the side chain; however, charged residues such as arginine and aspartic acid severely retard the reaction rate, and proline appears to virtually block the cleavage. Based on the limited number of peptides studied, a rather surprising observation is that in contrast to other similar hydrolases (1, 6, 7), the muscle enzyme does not appear to be affected in a major way by the length of the peptide substrate. If this can be confirmed with a broader spectrum of longer peptides, this enzyme could be a useful tool in protein sequencing of acetylated proteins. The fact that the enzyme will also cleave N-formylamino acids from formylated peptides (Table II) extends this possible use to prokaryotic proteins as well.

**What is the relationship of the muscle acetylaminoacyl-peptide hydrolyase to other similar hydrolases?** Kobayashi and Smith (2) have discussed some of the key properties of different preparations of mammalian hydrolases and pointed out differences in specific activity, specificity, apparent molecular weight, and effects of protein reagents and metal ions. There are obvious similarities as well as obvious differences in these parameters, and it is consequently difficult at this stage of knowledge to draw any firm conclusions regarding the relatedness of the enzymes. Perhaps the most important parameter, the substrate specificity, is the most difficult one to assess, since the selection of substrates and the assay methods vary so widely for the different studies. Our enzyme should be identical to the one reported in rat and rabbit skeletal muscle (5), but both the \(M_c\) and the activity toward Ac-Cys peptides in particular are significantly different. Further studies will be needed to resolve this issue.

Is it possible that the original hydrolyase extracted from ribosomes and claimed to be specific for Ac-Met-peptides (8) is different from the one reported here purified from whole muscle extract? All our data suggest that the two are the same enzyme and, as we suggested in the original report, that the association with ribosomes is an artifact probably of no relevance to the \textit{in vivo} function of the enzyme. Calculating back from the total purification, the specific activity of the ribosomal preparation and of the pure enzyme, it is clear that only 1-3% of the ribosomes would be associated with the

### Table I

**Purification of hydrolyase from 800 g of rabbit muscle**

| Purification step | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Recovery (%) | Purification |
|-------------------|--------------------|------------------------|----------------------------|-------------|--------------|
| 1. Homogenate     | 26,540             | 4,350                  | 0.16                       | 1           | 1            |
| 2. Ammonium sulfate fractionation (20-40%) | 3,597 | 3,655 | 1.0 | 84 | 6 |
| 3. Heat supernatant | 321.2            | 2,002                  | 6.2                        | 46          | 36           |
| 4. Gradient DE-52 chromatography | 9.95 | 1,501 | 151 | 34.5 | 900 |
| 5. Sephacryl S-300 chromatography (i) | 1.2 | 1,219 | 1,016 | 28 | 6,200 |
| 6. Sephacryl S-300 chromatography (ii) | 0.82 | 957 | 1,172 | 22 | 7,150 |

*One unit of enzyme activity is defined as the amount of enzyme that will convert 5% (10 mol) of the substrate to product under standard conditions (200 nmol of Ac-Met-Leu-Gly in 200 μl of buffer A, 30 min at 25 °C).*
The reactions were all carried out at 25 °C in 50 mM sodium phosphate buffer, pH 6.9, containing 2 mM MgCl₂ and 1 mM EDTA. The substrate concentration was established by amino acid analysis and was 0.85–1.15 mM except in a few cases; these are noted by giving the actual concentration in parentheses. Standard conditions used for the better substrates were 0.5 μg of enzyme and 30 min incubation time. For the poorer substrates, the amount of enzyme and the incubation time was increased to 2.5 μg and/or 2 h, respectively. The observed rates were then normalized to standard conditions. When possible, the peptides have been grouped to facilitate direct comparison of a given structural feature. Some peptides, marked with an asterisk, are consequently listed more than once.

### Table II

**Substrate quality of acylpeptides for acylaminocarboxylic acid hydrolase**

The reactions were all carried out at 25 °C in 50 mM sodium phosphate buffer, pH 6.9, containing 2 mM MgCl₂ and 1 mM EDTA. The substrate concentration was established by amino acid analysis and was 0.85–1.15 mM except in a few cases; these are noted by giving the actual concentration in parentheses. Standard conditions used for the better substrates were 0.5 μg of enzyme and 30 min incubation time. For the poorer substrates, the amount of enzyme and the incubation time was increased to 2.5 μg and/or 2 h, respectively. The observed rates were then normalized to standard conditions. When possible, the peptides have been grouped to facilitate direct comparison of a given structural feature. Some peptides, marked with an asterisk, are consequently listed more than once.

| Substrate | 10⁻⁶ × k (min⁻¹) | Substrate | 10⁻⁶ × k (min⁻¹) |
|-----------|-----------------|-----------|-----------------|
| *Ac-TGG  | 4689            | Ac-CAAGVC (reduced) | 60 |
| *Ac-AGG  | 2625            | Ac-CTFQCNPRG (reduced) | 51 |
| *Ac-MGG  | 2193            | Ac-YAR (2.1 mM) | 53 |
| *Ac-NGG  | 828             | Ac-DSQPR | 22 |
| Ac-VGG (1.8 mM) | 16 | Ac-FGPETP | 7 |
| Ac-FGG  | 10              | Ac-PDASV | 4 |
| Ac-IGG (1.7 mM) | 4 | Ac-LGL | 3 |
| Ac-LGG  | 2               | Ac-ETP | 0 |
| Ac-RGG  | <1              | Ac(EA)₅ (6:4) | 0 |
| Ac-HGG  | <1              | Ac-RFA | 0 |
| Ac-EGG  | <1              | Ac-HLM | 0 |
| Ac-PGG  | <1              | Ac-DRVYHPF | 0 |
| *Ac-MLG | 2023            | Ac-D₅ₐ (M, 2500-6000) | 0 |
| *Ac-SLG  | 1375            |               |    |
| *Ac-GLG | 511             | Formyl-MLG | + |
| Ac-LLG  | 1               | Formyl-MLE | + |
| Ac-PLG  | <1              | Formyl-MM | + |
| Ac-MLG  | 2193            | Ac-AAA (0.79 mM) | 3798 |
| Ac-MAM  | 2148            | Ac-ALA | 2699 |
| Ac-MAM  | 2112            | Ac-ALA | 2581 |
| Ac-MGG  | 2396            | Ac-MGG | 9 |
| Ac-MPG  | 2212            | Ac-MPG | 7 |
| Ac-MGG  | 2581            | Ac-ML | 9 |
| Ac-MDF  | 756             | Ac-ML | 3798 |
| Ac-MRG  | 29              | *Ac-GGG | 2396 |
| Ac-MPG  | 9               | Ac-GGG | 906 |
| *Ac-MLG | 2023 | Ac-GGG | 3798 |
| *Ac-TGG | 4689 | Ac-MLG | 906 |
| Ac-TVG  | 4378            | Ac-MLG | 3798 |

**Effect of NH₂-terminal acetylamino acid**

- **A. Effect of NH₂-terminal acetylamino acid**
  - *Ac-TGG: 4689, Ac-CAAGVC (reduced) 60
  - *Ac-AGG: 2625, Ac-CTFQCNPRG (reduced) 51
  - *Ac-MGG: 2193, Ac-YAR (2.1 mM) 53
  - *Ac-NGG: 828, Ac-DSQPR 22
  - Ac-VGG (1.8 mM): 16, Ac-FGPETP 7
  - Ac-FGG: 10, Ac-PDASV 4
  - Ac-IGG (1.7 mM): 4, Ac-LGL 3
  - Ac-LGG: 2, Ac-ETP 0
  - Ac-RGG: <1, Ac(EA)₅ (6:4) 0
  - Ac-HGG: <1, Ac-RFA 0
  - Ac-EGG: <1, Ac-HLM 0
  - Ac-PGG: <1, Ac-DRVYHPF 0
  - *Ac-MLG: 2023, Ac-D₅ₐ (M, 2500-6000) 0
  - *Ac-SLG: 1375
  - *Ac-GLG: 511, Formyl-MLG +
  - Ac-LLG: 1, Formyl-MLE +
  - Ac-PLG: <1, Formyl-MM +
  - Ac-MLG: 2193, Ac-AAA (0.79 mM) 3798
  - Ac-MAM: 2148, Ac-ALA 2699
  - Ac-MAM: 2112, Ac-ALA 2581
  - Ac-MGG: 2396, Ac-MGG 9
  - Ac-MFG: 2755, Ac-APG 7
  - *Ac-MLG: 2023, Ac-APG 9
  - Ac-MDF: 756
  - Ac-MRG: 29, *Ac-GGG 828
  - Ac-MPG: 9, Ac-GGG 713
  - *Ac-MLG: 2023, Ac-GGG 522

**Effect of second and third residue**

- *Ac-MGG: 2193, *Ac-AGG: 2625
- Ac-MAM: 2148, Ac-MAM: 2396
- Ac-MAM: 2112, *Ac-Ala 2581
- Ac-MFG: 2755, Ac-APG 7
- *Ac-MLG: 2023, *Ac-MLG 9
- Ac-MDF: 756
- Ac-MRG: 29, *Ac-GGG 828
- Ac-MPG: 9, Ac-GGG 713
- *Ac-MLG: 2023, *Ac-MLG 522

**Effect of peptide length**

- Ac-AA: 478, Ac-MA 373
- *Ac-AAA (0.79 mM): 3798, *Ac-MAM: 2396
- Ac-AAA: 2212, *Ac-MAGGDASGE 906
- Ac-AAA (0.76 mM): 1456, Ac-MG: 233
- *Ac-AAGGDASGE (0.65 mM): 1719, *Ac-MG: 2148
- *Ac-ALA: 2581, Ac-ML 234
- Ac-ALGA: 1842, *Ac-MLG 2023
- *Ac-SLG: 1375, Ac-MDETGDALVA (0.47 mM) 1034
- Ac-SGAGA: 338, *Ac-GAGGDASGE 136

The biological function of the enzyme is not known. In our original report we proposed two possible specific roles for the enzyme: in the removal of NH₂-terminal Ac-Met from actin (12) and from aldolase (13) during normal biosynthetic processing. Neither of these roles now seem plausible. The NH₂-terminal methionine of muscle actin appears to be removed cotranslationally as free methionine, yielding after acetylation an intermediate Ac-Cys-Asp-sequence from which Ac-Cys must be removed to give, after a second acetylation step, the final Ac-Asp-NHz terminus of the processed actin. Since Ac-Cys is a poor substrate for the present hydrolase, there is no basis for considering the enzyme as a participant in actin biosynthesis. In the case of aldolase, the observation that muscle aldolase isolated in the presence of high concentration of tosylfluoride contained the NH₂-terminal sequence of Ac-Met-Pro-, whereas the normal preparations in the absence of the protease inhibitor yields aldolase with a free proline at the NH₂ terminus (13), seemed to present an obvious involve-
N-Acylaminoacyl-peptide Hydrolase from Rabbit Muscle

11079

ment for the present hydrolase, especially since the enzyme is inhibited by high concentrations of tosylfluoride. However, the specificity data presented here, showing that the enzyme is essentially inactive on an Ac-Met-Pro-substrate, seems to preclude any involvement of the hydrolase in aldolase processing as well.

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Supplemental Material to:

PURIFICATION AND CHARACTERIZATION OF AN ACETYLATED-AMINOACYL-PEPTIDE HYDROLASE FROM RABBIT MUSCLE

by C. Radhakrishna and Finn Wold

EXPERIMENTAL PROCEDURES

Materials. Young rabbit muscle was purchased from Pel-Freez. Most of the peptides were obtained from Research Plus Inc., Peninsula Laboratories Inc., Bachem Inc. and U.S. Biochemicals. Acetyl amino acid with the code was used and was purchased from MorphoTech, Bachem Inc. The following buffer solutions: acetic acid, trichloroacetic acid, sodium chloride, and acetic anhydride, were all purchased from Lachema. Acetyl Boc-aminocarbonyl chloride, acetyl amino acid, acetyl anhydride, and acetic acid were all obtained from Fluka. All other reagents were from Sigma. The purity of these reagents was determined by thin-layer chromatography. All solutions were prepared in distilled water.

Procedure for purifying and characterizing the activity of the enzyme. The purified enzyme was subjected to a thorough screening for any contaminating peptide activity. The purified enzyme was subjected to a thorough screening for any contaminating peptide activity. The purified enzyme was subjected to a thorough screening for any contaminating peptide activity.

Quantitative assay. For quantification of hydrolysis activity [14C]-Ac-Ac peptides were used under the same conditions as those described above. The reaction mixture was incubated with [14C]-Ac-Ac peptides and subjected to the same purification and characterization procedures as described for the purified enzyme. The specific activity of the purified enzyme was determined by the method of Lowry et al. (1951) and expressed as units of activity per milligram of protein.

RESULTS

Organ culture: All the steps described below were carried out at 4°C. Young rabbit muscle was washed overnight at 4°C, rinsed thoroughly and suspended in twice the volume of buffer A (50 mM NaCl, 1 mM MgCl₂, 1 mM EDTA) with 0.5 g of enzyme and 0.5 g of substrate.

Peptide competitive assay. Unlabeled Ac-Nle-Tyr-Lys was used as substrate, and the reaction was terminated by heating in boiling water bath for 5 min. After centrifugation, the supernatant solutions were lyophilized and redissolved in 20 ml of buffer A and 0.5 g of enzyme was added. The mixture was incubated with the substrate and the reaction was terminated by heating in boiling water bath for 5 min. The reaction mixture was used as substrate for the purified enzyme. The enzyme activity was determined by the method of Lowry et al. (1951) and expressed as units of activity per milligram of protein. The specific activity of the purified enzyme was determined by the method of Lowry et al. (1951) and expressed as units of activity per milligram of protein.
N-Acylaminoacyl-peptide Hydrolase from Rabbit Muscle

**TABLE III**

| Peptide | Concentration (μM) | Relative Activity (%) |
|---------|--------------------|-----------------------|
| Urea    | 50                 | 60.1                  |

Retention Time (min)

The enzyme was precipitated with various reagents for 30 min at room temperature in the absence of substrate under standard assay conditions as described in Experimental Procedures. The assay was then initiated by the addition of 0.5 μM of a concentrated (40 μM) solution of the substrate Ac-Asp-Leu. After the stock enzyme solution containing metal ions, the samples used to evaluate the effect of metal ions were dialyzed using a Sephadex G-10 column (1 x 10 cm) which had previously been treated with dialysis, Polyethylene glycol 10 kDa, and then extensively washed with glass distilled water. Fractions containing inactivating activity were pooled and assayed as above.

**Abbreviations:**
- TFA: Trifluoroacetic acid
- THF: Tetrahydrofuran
- DMF: Dimethylformamide
- N,N-dimethylacetamide
- p-DMS: p-Dimethylsulfoxide

**Fig. 1**

The enzyme profile of about 200 μM of the reaction products of the octapeptide A (Ac-Lys-Pro-Leu-Arg-Glu-Glu-Leu-Asp) with the monomethyl esters of Ac-Asp (A), Ac-Arg (B) and Ac-Leu (C). The peaks labeled 1 is the peptide A, that labeled 2 Ac-Asp (A), Ac-Arg (B) and Ac-Leu (C) respectively. Peaks 3 and 4 represents acetylated nonapeptide products. Peaks 3 and 4 from 1 each gave a single peak of about 300 μM each (330.2 and 330.3, respectively), on FAB-MS (calculated for [Ac-Leu*3] 836.5), the unreacted mixture of peaks 3 and 4 from 1 gave a single peak of mass 376.3 (calculated for [Ac-Leu*3] 376.3). Component 3 is the tripeptide products with three 

**Fig. 2**

The enzyme profile of about 200 μM of the reaction products of the octapeptide A (Ac-Lys-Pro-Leu-Arg-Glu-Glu-Leu-Asp) with the monomethyl esters of Ac-Asp (A), Ac-Arg (B) and Ac-Leu (C). The peaks labeled 1 is the peptide A, that labeled 2 Ac-Asp (A), Ac-Arg (B) and Ac-Leu (C) respectively. Peaks 3 and 4 represents acetylated nonapeptide products. Peaks 3 and 4 from 1 each gave a single peak of about 300 μM each (330.2 and 330.3, respectively), on FAB-MS (calculated for [Ac-Leu*3] 836.5), the unreacted mixture of peaks 3 and 4 from 1 gave a single peak of mass 376.3 (calculated for [Ac-Leu*3] 376.3). Component 3 is the tripeptide products with three 

**Fig. 3**

The enzyme profile of about 200 μM of the reaction products of the octapeptide A (Ac-Lys-Pro-Leu-Arg-Glu-Glu-Leu-Asp) with the monomethyl esters of Ac-Asp (A), Ac-Arg (B) and Ac-Leu (C). The peaks labeled 1 is the peptide A, that labeled 2 Ac-Asp (A), Ac-Arg (B) and Ac-Leu (C) respectively. Peaks 3 and 4 represents acetylated nonapeptide products. Peaks 3 and 4 from 1 each gave a single peak of about 300 μM each (330.2 and 330.3, respectively), on FAB-MS (calculated for [Ac-Leu*3] 836.5), the unreacted mixture of peaks 3 and 4 from 1 gave a single peak of mass 376.3 (calculated for [Ac-Leu*3] 376.3). Component 3 is the tripeptide products with three 

**Fig. 4**

The enzyme profile of about 200 μM of the reaction products of the octapeptide A (Ac-Lys-Pro-Leu-Arg-Glu-Glu-Leu-Asp) with the monomethyl esters of Ac-Asp (A), Ac-Arg (B) and Ac-Leu (C). The peaks labeled 1 is the peptide A, that labeled 2 Ac-Asp (A), Ac-Arg (B) and Ac-Leu (C) respectively. Peaks 3 and 4 represents acetylated nonapeptide products. Peaks 3 and 4 from 1 each gave a single peak of about 300 μM each (330.2 and 330.3, respectively), on FAB-MS (calculated for [Ac-Leu*3] 836.5), the unreacted mixture of peaks 3 and 4 from 1 gave a single peak of mass 376.3 (calculated for [Ac-Leu*3] 376.3). Component 3 is the tripeptide products with three
**N-Acylaminoacyl-peptide Hydrolase from Rabbit Muscle**

Fig. 2. Illustrations of the two HPLC procedures used in the quantitative activity assay. A is the routine program (linear gradient from 0 to 40% acetonitrile in 30 min, followed by a gradient from 40 to 85% acetonitrile in 5 min and a 10 min return to and wash with the starting buffer) illustrated with the release of [15O2]Ac-Met from [15O2]Ac-Met-Leu-Gly. B is the modified program used for Ac-Cly, Ac-Ala, Ac-Ser, Ac-Arg, Ac-Thr peptides in which a 5 min isocratic elution at 5% acetonitrile precedes the routine program. It is illustrated with the release of Ac-Ala from Ac-Ala-Leu-Gly. The program in A causes elution of these more hydrophilic products in fractions 10-15 relative to acetate at 9; program B gives improved resolution with elution in fractions 13-19 relative to acetate at 11. A represents substrate and acetylamino acid product, [15O2]; B = acetylamino acid internal marker (PM).

Fig. 3. Fractionation of the test experiment by chromatography on DE-52. About 90% of the total protein applied to the column was eluted in the initial wash with the starting buffer (not shown in the figure). Enzyme activity is indicated by the broken line and protein content (at 280 nm absorbance) by the solid line. The experimental details are given in the text.

Fig. 4. Gel filtration on Sepharose 6B-300 of the pooled and concentrated active fractions from DE-52 chromatography (A) and of the pooled and concentrated activity peak from A (B). The column was calibrated with known M, references; their elution positions are indicated with arrows in B. From left to right they are blue dextran (void volume) bovine thyroglobulin (670,000), bovine γ-globulin (158,000), bovine serum albumin (66,000), chicken ovalbumin (45,000) and horse myoglobin (17,000). The M, of the hydrolase estimated relative to these markers is 230,000-245,000. The insert in B shows the specific activity (mg units/mg protein) across the protein-activity peak. The experimental details are given in the text.

Fig. 5. SDS-PAGE of 12 μg of the purified hydrolase (A). The M, markers in B are myosin (200,000), E. coli β-galactosidase (116,000), rabbit muscle phosphorylase b (92,500), bovine serum albumin (66,000) and chicken ovalbumin (45,000).

Fig. 6. Effect of pH on the activity of hydrolase on the substrate Ac-Met-Leu-Gly. The results were obtained after 30 min incubation at 25°C with 1 μg of enzyme and 200 μM of substrate in 200 μl of 50 mM Na-acetate buffer (pH 5.0), Na-phosphate buffer (pH 6.0 and 6.9) or Tris/HC1 buffer (pH 8.0). All buffers contained 2 mM MgCl2 and 1 mM EDTA.