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CONTAMINATION OF DFP-TREATED LEUCINE AMINOPEPTIDASE WITH MULTIPLE ENDOPEPTIDASES

L. J. DEFTOS* AND J. T. POTTS*, JR.

Section on Polypeptide Hormones, Laboratory of Molecular Diseases, National Heart Institute, National Institutes of Health, Bethesda, Md. (U.S.A.)

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SUMMARY

1. Commercial preparations of leucine aminopeptidase (EC 3.4.1.1) were found to be contaminated with multiple endopeptidases of high molecular weight and varying substrate specificity.

2. These contaminating endopeptidases could not be completely eliminated from the leucine aminopeptidase preparations with endopeptidase inhibitors or by further purification procedures.

3. These findings emphasize the importance of testing for non-specific proteases in any preparation of leucine aminopeptidase to be used for the limited digestion of proteins or peptides.

INTRODUCTION

Three commercially available preparations of leucine aminopeptidase[1] were found to be contaminated with endopeptidases to an extent that precluded their use to achieve sequential degradation of proteins. The endopeptidases were of high molecular weight and differing substrate specificity. These contaminants could not be eliminated by either treatment of the enzyme with endopeptidase inhibitors or by subjecting the preparations to several further purification procedures. These findings impose serious limitations on the use of many currently available commercial preparations of leucine aminopeptidase.

MATERIALS AND METHODS

Leucine aminopeptidase preparations were obtained from three commercial sources, (Worthington Biochemical Corporation, N.J.; Mann Research Laboratories, N.Y.; Boehringer Mannheim Corporation, Germany). Aminopeptidase activity was measured by a modification of the method of BINKLEY AND TORRES[2]. The substrate for the aminopeptidase activity was 0.05 M leucineamide (Cyclo Chemical Corporation, Calif.). Digestions were performed in 0.05 M Tris buffer (pH 8.5), made

* Present address: Endocrine Unit, Massachusetts General Hospital, Boston, Mass. U.S.A.

0.005 M in $MgCl_2$ (Fisher Scientific Company, N.J.). Specific activity was expressed as the rate of change in absorbance of the substrate per mg of enzyme. Absorbance change at 232 mμ was measured in an ultraviolet spectrophotometer (Beckman DB) equipped with a logarithmic recorder (Photovolt). Incubations were performed at 25° in cuvettes with 1-cm light paths.

The principal proteins used to test for endopeptidase activity, as suggested by FRATER, LIGHT AND SMITH[3], were horse heart cytochrome *c* and mercuripapain (Nutritional Biochemicals, Ohio). These proteins are resistant to aminopeptidase activity (cytochrome *c* has an *N*-acetylglycine and mercuripapain a penultimate proline, respectively, at the amino terminus). These proteins were incubated with leucine aminopeptidase in the Tris buffer at 40° for 18–24 h at a molar substrate to enzyme ratio of 500:1. Pure bovine parathyroid hormone[4] and ribonuclease S protein[5] were also incubated under the same conditions. In control experiments identical concentrations of substrate and enzyme were incubated separately. Aliquots of leucine aminopeptidase were treated with a 1000-fold molar excess of DFP (Aldrich Chemical Corporation, Wisc.) at 4° for 18 h. Prior to use each vial of DFP was first assayed to insure adequate potency as previously described[6]. Aliquots of leucine aminopeptidase were also treated with a 50-fold molar excess of iodoacetic acid (Eastman Kodak Corporation, N.Y.) in Tris buffer (pH 7.5) for 2 h at 4° at a final concentration of iodoacetic acid of 0.025 M. Amino acid analyses were performed with a Beckman 120B automatic amino acid analyzer. Amino terminal amino acids were analyzed by the SANGER procedure[7].

Sephadex G-100 (Pharmacia, Sweden) and Sagarose 6 (Seravac, Great Britain) were heated for 3 h in boiling water and then resuspended to remove fine particles. DEAE-cellulose (DE-52 Whatman) was prepared for chromatography by pre-cycling in acid and base. The DEAE-cellulose column (1 cm × 10 cm) was equilibrated with the Tris buffer and developed with a linear gradient of NaCl (Limit concentration 0.2 M, total volume 70–80 ml). The Sephadex and Sagarose columns (each 1 cm × 100 cm) were developed with Tris buffer. 15 mg of leucine aminopeptidase dissolved in the Tris buffer were applied to each column. In one gel filtration experiment on Sagarose the enzyme was applied to the column in Tris buffer made 8 M in urea.

Disc gel electrophoresis was performed by a modification of previously described methods[8]. The monomer concentration (w/v) was 4%; the gel pH was 2.7. Highly purified leucine aminopeptidase was prepared from kidneys of Yorkshire hogs as described by HIMMELHOCH AND PETERSON[9] and was also incubated with the test proteins.

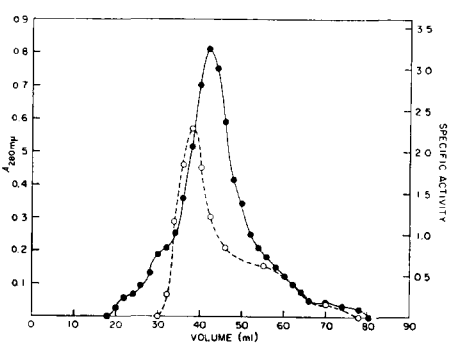
RESULTS

Each of the commercially available preparations of leucine aminopeptidase tested was found to be contaminated with endopeptidase. Amino acids were released from cytochrome *c* and mercuripapain even at a molar ratio of substrate to enzyme of 500:1. The substrate and enzyme blanks which accompanied each experiment did not contain any detectable amino acids. To eliminate the possibility that either the test proteins were contaminated with other proteins that were susceptible to aminopeptidase digestion, or that non-enzymic cleavages occurred in the test substrates during incubation, amino terminal groups were determined by the SANGER procedure[7]

after incubation of the cytochrome *c* and mercuripapain without enzyme. Only isoleucine was detected with mercuripapain and no amino acid derivative was found with cytochrome *c*. This confirmed that the amino acids released during incubation of the peptides resulted from endopeptidase contaminants that had produced internal bond cleavages, thereby providing sites susceptible to degradation by leucine aminopeptidase.

Treatment of each of these preparations of leucine aminopeptidase with DFP and iodoacetic acid did not eliminate endopeptidase activity, although the relative ratio of amino acids released was altered. These results indicated that multiple proteases were present in the preparations, some of which were sensitive to the protease inhibitors. Since the endopeptidase activity could not be completely eliminated by treatment with protease inhibitors, two enzyme preparations (Worthington) were subjected to further purification procedures. The earliest preparation received from Worthington had not been fractionated on DEAE-cellulose. All of the enzymic activity of this preparation, when applied to a DEAE-cellulose column, was recovered by elution with a NaCl gradient. The results (Fig. 1) indicated that the leucine aminopeptidase constituted only a small fraction of the total protein. Specific activity of the recovered enzyme was increased 5-fold over that of the starting material, but all fractions containing leucine aminopeptidase were still contaminated with endopeptidase. During the course of these studies, Worthington provided us with a preparation which had been fractionated on DEAE-cellulose. Chromatography of this fraction under identical conditions indicated that the enzyme now constituted a larger fraction of the preparation; there was only a slight increase in specific activity (Fig. 2). Although the preparation therefore seemed more purified, endopeptidase activity was still found in all fractions of the leucine aminopeptidase recovered.

When this more highly purified preparation of enzyme was subjected to gel filtration on Sephadex G-200, considerable size heterogeneity was evident in the preparation. The leucine aminopeptidase eluted at a K_d of 0.10. However, again, no fraction containing leucine aminopeptidase was free of endopeptidase activity; at least some of the endopeptidase activity was DFP and iodoacetic acid insensitive.



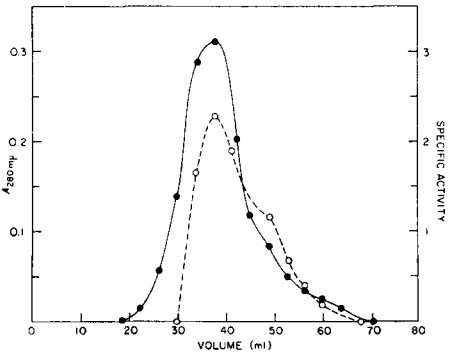


Fig. 1. DEAE-cellulose chromatography of a commercial preparation of leucine aminopeptidase. 15 mg of protein were applied to the column (1 cm × 10 cm) and eluted with a linear gradient of NaCl, limit concentration 0.2 M. ○— — —○, specific activity of the leucine aminopeptidase; ●—●, absorbance of the effluent protein.

Fig. 2. DEAE-cellulose chromatography of a more highly purified preparation of leucine aminopeptidase. Conditions as in Fig. 1.

Further gel filtration was performed on Sagarose, using a gel concentration (Sagarose 6) chosen to provide optimal purification of the enzyme (elution K_d 0.5). The enzyme activity was separated from the bulk of the protein in the preparation (Fig. 3). The main peak of protein did elute at a K_d of 0.5; the leucine aminopeptidase eluted on the ascending limb of this protein peak. However, each fraction containing aminopeptidase activity was again found to contain endopeptidase activity. This result suggested that some of the endopeptidases were similar in molecular weight to the leucine aminopeptidase. To eliminate the possibility that the contaminating endopeptidases were non-covalently bound to the leucine aminopeptidase, another aliquot of the DEAE-cellulose purified enzyme was applied to the Sagarose 6 column in the presence of 8 M urea to minimize any protein–protein interaction. However, the elution pattern was not significantly changed and all fractions of the leucine aminopeptidase still contained endopeptidase activity.

Further evidence was obtained by study of the more purified preparation of

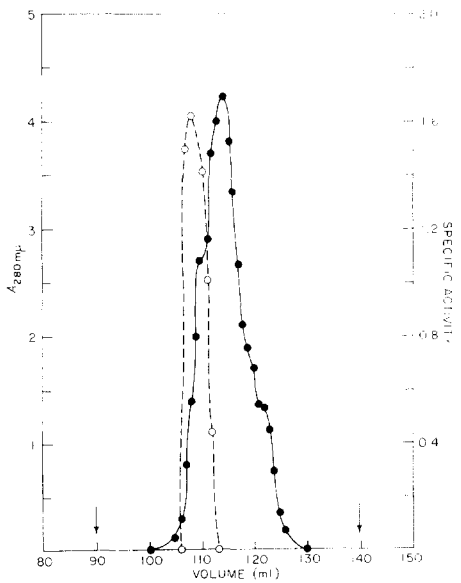


Fig. 3. Gel filtration on Sagarose 6 of 15 mg of leucine aminopeptidase purified by DEAE-cellulose chromatography (Fig. 2). The column (1 cm × 100 cm) was developed with 0.05 M Tris buffer (pH 8.5). ○— — —○, specific activity of the leucine aminopeptidase; ●—●, absorbance of the effluent proteins. Arrows indicate the excluded and salt volumes, respectively, of the column.

Fig. 4. Disc gel electrophoresis of the leucine aminopeptidase after DEAE-cellulose chromatography and Sagarose 6 gel filtration (105–115-ml fraction of Fig. 3). Gel conditions: 4% monomer concentration (w/v) (pH 2.7).

leucine aminopeptidase that multiple endopeptidase contaminants were present. Fractions from the Sagarose 6 column (Fig. 3) which eluted later than the leucine aminopeptidase (at 115–120 ml) were found to contain endopeptidase with specificity different from that of the proteases that co-eluted with the leucine aminopeptidase fraction (105–115 ml). Table I contrasts the relative quantities of amino acids released from the cytochrome *c* when incubated under identical conditions with the 105–115-ml *versus* the 115–120-ml fraction of the Sagarose 6 column. To eliminate the possibility that the endopeptidase still contaminating the leucine aminopeptidase fraction was a protease completely sensitive to inhibitors, this fraction was treated with DFP and iodoacetic acid and then incubated with cytochrome *c*. The results demonstrated in Table I show that, despite DFP and iodoacetic acid treatment, the purified leucine aminopeptidase fraction still released amino acids, although the relative ratios of free

TABLE I

AMINO ACIDS RELEASED FROM CYTOCHROME *c* BY DIFFERENT PREPARATIONS OF LEUCINE AMINOPEPTIDASE

	*μmoles/μmole**			*Ratios*	
	A	*B*	*C*	*B/A*	*C/A*
Asp	1.60	0.16	0.00	0.10	0.00
Thr	0.75	0.53	0.25	0.70	0.33
Glu	0.54	0.39	0.25	0.70	0.46
Pro	1.00	0.39	0.08	0.39	0.08
Gly	0.36	0.71	0.20	1.97	0.56
Ala	0.30	0.08	0.35	0.27	1.17
Val	0.40	0.69	0.00	1.72	0.00
Met	0.00	0.19	0.00	—	0.00
Ile	0.21	0.43	0.00	2.04	0.00
Leu	0.34	0.74	0.00	2.18	0.00
Tyr	0.13	0.31	0.00	2.38	0.00
Phe	0.26	0.53	0.00	2.04	0.00

* Amino acids released during incubation of cytochrome *c* with fractions of leucine aminopeptidase purified by gel filtration on Sagarose 6 (Fig. 3). Results, based on digestion of 1 mg/ml of cytochrome *c*, are expressed as μmoles of amino acid/μmole of substrate. Column A lists the amino acids released when the 105–115-ml fraction from the column was used; Column B, the results after this fraction had been treated with DFP and iodoacetic acid; Column C, amino acids released when the 115–120-ml fraction was used.

amino acids were altered by these inhibitors. The persistent heterogeneity of this purified fraction of leucine aminopeptidase was confirmed by disc gel electrophoresis which demonstrated multiple bands (Fig. 4).

An aliquot of this leucine aminopeptidase (purified on Sagarose 6 and treated with DFP and iodoacetic acid) was incubated with ribonuclease S protein and bovine parathyroid hormone. Amino acids were released which were not in accord with the amino terminal structure of these molecules*.

* A preparation of Worthington leucine aminopeptidase had been used at an earlier time in this laboratory to achieve limited digestion of proteins. This earlier preparation did not release any amino acids from ribonuclease S protein. Furthermore, the amino acids released from bovine parathyroid hormone were in accord with the known amino terminal sequence of this polypeptide[4]. Hence, there has apparently been variation in the extent of endopeptidase contamination in commercial preparations of the enzyme in the past.

Through the courtesy of HIMMELHOCH and PETERSON[9] we were able to prepare and test a preparation of leucine aminopeptidase extracted from kidneys of a uniform strain of hogs. This leucine aminopeptidase with a very high specific activity did not release any amino acids from cytochrome *c*, mercuripapain, or ribonuclease S protein. Furthermore, it released amino acids from bovine parathyroid hormone that were in accord with the known amino terminal structure of this molecule[4].

DISCUSSION

These studies demonstrate that all three commercially available preparations of leucine aminopeptidase tested were contaminated with endopeptidase activity. In agreement with FRATER, LIGHT AND SMITH[3], we found that some of the endopeptidase activity of these enzyme preparations was insensitive to DFP However, the preparations currently available to us also contained endopeptidase activity that was not inhibited by treatment with iodoacetic acid.

The results found during further purification and treatment of the enzyme preparations indicated that at least three contaminating endopeptidases were present. These endopeptidases varied in molecular weight, substrate specificity, and sensitivity to protease inhibitors. Some of them were similar in charge and size to leucine aminopeptidase. Endopeptidase activity could not be eliminated by chromatography on DEAE cellulose. One endopeptidase eluted later from Sagarose 6 than the aminopeptidase; however, at least two of the contaminants co-eluted with leucine aminopeptidase and, therefore, seemed to be similar to the aminopeptidase in molecular weight or apparent molecular volume. Furthermore, at least one of these two persistent contaminants could not be inhibited by the combination of DFP and iodoacetic acid. Hence, despite the combination of purification on DEAE-cellulose, gel filtration on Sephadex and Sagarose, and finally, exhaustive treatment with DFP and iodoacetic acid, a satisfactory endopeptidase-free preparation of leucine aminopeptidase could not be obtained.

By contrast, a highly active leucine aminopeptidase preparation prepared by us according to the method of HIMMELHOCH AND PETERSON[9] was found to be free of significant endopeptidase activity. This serves to emphasize the importance of this new procedure for leucine aminopeptidase purification; the endopeptidase activity, difficult to eliminate from commercial preparations of the enzyme, is removed early in this new fractionation method[9].

The persistence of endopeptidases in several preparations of commercially purified leucine aminopeptidase makes it imperative to use sensitive tests for nonspecific protease activity in any sample of leucine aminopeptidase to be used for limited modification of proteins or peptides.

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