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DETECTION AND ANALYSIS OF NEUTRAL ENDOPEPTIDASE FROM TISSUES WITH SUBSTRATE GEL ELECTROPHORESIS

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Neutral endopeptidase from human or bovine tissues retains enzymatic activity following electrophoresis and immobilization in polyacrylamide gels. Infiltration of the gel with a fluorogenic substrate permits identification of the active enzyme by fluorescence associated with a distinct protein band. This technique both separates and identifies the enzymatically active species from a crude cell membrane fraction or from partially purified extracts that contain contaminating proteins. Enzymatic activity is quantitated by photographing the fluorescent bands and scanning the negatives with a laser densitometer. Because as little as 25 ng of enzyme can be detected by this method, it could be used where the amount of material is limited.

Neutral endopeptidase (E.C.3.4.24.11, NEP) or enkephalinase is found in many different mammalian tissues, including the central nervous system, kidney, lung, intestine and the male genital tract (1). It is an integral membrane protein of epithelium in various organs attached to plasma membrane by an N-terminal anchor peptide (2). Neutral endopeptidase is also active in lymph nodes (3), cultures of human lung fibroblasts (4) and the surface membrane of polymorphonuclear neutrophils (5,6). A recent report identified the human Common Acute Lymphoblastic Leukemia Antigen (CALLA) as neutral endopeptidase (7).

The physiologic role of this ubiquitous enzyme can not be ascertained from its tissue distribution, but its activity on numerous small peptide substrates suggests that it may regulate peptide-ligand interactions at cell surfaces (1,3).

Most methods for identification of neutral endopeptidase in tissues depend upon either immunologic detection or measurement of enzymatic activity.

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Immunohistochemical methods, which provide unambiguous identification by specific polyclonal or monoclonal antibodies, were used to detect the enzyme in a variety of tissues (3,4,8,9), but these methods do not provide quantitative determinations of the enzymatic activity. Activity can be measured biochemically, using various synthetic or natural peptide substrates, but in tissue or cell extracts these assays are sometimes limited by the presence of other enzymes or inhibitors.

This report describes the combination of polyacrylamide gel electrophoresis with enzymatic cleavage of a dansylated peptide substrate to separate and identify enzymatically active neutral endopeptidase isolated from human kidney and from bovine neck ligament. This particular method offers the advantage of simultaneous separation of molecular weight species and quantitation of neutral endopeptidase activity in tissue samples.

MATERIALS AND METHODS

Enzyme purification

Neutral endopeptidase was partially purified from membrane fractions isolated from human kidney and from nuchal ligament of bovine fetuses. Human kidney tissue was an autopsy specimen that was obtained within 4 hrs postmortem. Fetal bovine ligament tissue was dissected immediately following death at a commercial slaughter house. Thirty gm of tissue was homogenized (5 volumes/gm tissue) in 50 mM MES (2[N-morpholine]ethanesulfonic acid) buffer (pH 6.5) containing 0.1 mM PMSF. The homogenate was centrifuged at 3,500 x g for 5 min at 4^{o}, the pellet was re-extracted and the supernatant material pooled with that reserved from the first centrifugation.

The pooled supernatants were passed through a sintered glass filter and centrifuged at 100,000 x g for 1 hr. The pellet, which contains primarily cell membranes, was resuspended in 10 mM Tris HCl (pH 7.5) containing 0.1 mM PMSF and 0.5% CHAPS. This fraction was incubated overnight at 4^{o} then centrifuged at 100,000 x g for 1 hr to removed insoluble material. The CHAPS extract was concentrated 15 fold through an Amicon YM-30 membrane and applied to a mono Q FPLC column. The material was fractionated using a 0-500 mM NaCl gradient containing 10 mM Tris and 0.5% CHAPS. The active fractions were pooled and concentrated by Amicon filtration. The proteins were separated by electrophoresis by electrophoresis standard Laemmli gels (10).

Enzyme activity

Neutral endopeptidase activity was measured by the method of Florentin et al (11) using a continuous recording assay of cleavage of N-dansyl-D-alanyl-glycyl-p-nitrophenylalanyl-glycine (DAGNPG) at a final concentration of 50 uM in 50 mM Tris HCl (pH 7.5). The fluorescent product was measured in a Perkin-Elmer photofluorometer using an excitation wave length of 342 and an emission of 562. Parallel samples contained phosphoramidon (1 uM), an inhibitor of neutral endopeptidase (1), and enzyme activity was expressed as the difference between uninhibited and inhibited samples. The amount of enzyme in each partially purified sample was estimated by comparison with the specific activity of purified human renal neutral endopeptidase (a gift from Dr. E.G. Erdos, The University of Illinois at Chicago). Protein was measured by the method of Bradford (12).

Substrate gels

Sustrate-impregnation was used to identify enzyme activity in protein bands separated by polyacrylamide gel electrophoresis. Enzymatically active fractions obtained from FPLC were applied in duplicate to standard 12% polyacrylamide gels (10). Following electrophoresis, SDS was removed from the gel by washing three times in 15 ml of 10 mM Tris HCl containing 0.5% CHAPS and then in Tris containing 0.5% CHAPS, 0.15 M NaCl and 0.3 M urea. The gel was then divided so that replicate samples could be incubated with substrate alone or combination with phosphoramidon. The gels were left overnight at 4^{o} in Tris/CHAPS buffer containing the dansylated substrate, DAGNPG, at a final concentration of 50 uM. Inclusion of 0.15 M NaCl and 0.3M urea in the buffer improved the sensitivity of detection but was not essential. Fluroescence commonly developed within 30 min at 4^{o} C, but overnight incubation increased intensity. Duplicate samples were incubated with phosphoramidon (1 uM).

The gels were photographed with a MP-4 polaroid camera system equipped with a 65A wratten gelatin filter and illuminated with a 304 nM trans-illuminator. Negatives were scanned using a Zenith laser densitometer, and intensity was quantitated in arbitrary units. Noise was reduced by combining traces from multiple scans, and background fluorescence was eliminated by subtraction of phosphoramidon-inhibited control lanes. Areas under the generated curves were compared to the amount of enzyme applied to the gels.

To confirm the position of enzyme activity, gels were run under standard conditions, washed as described above, and the lanes were cut into 21 mm slices. Each slice was assayed for neutral endopeptidase activity using 50 uM DAGNPG in Tris HCl (pH 7.5) with or without addition of phosphoramidon. Staining with Coomassie blue or silver nitrate indicated the corresponding protein band.

RESULTS AND DISCUSSION

Immobilized enzymes are commonly used in basic research and in industrial processes. Gel entrapment in polyacrylamide has been successfully used for a number of enzymes, including proteases (13). This method of immobilization appears to stabilize activity by protecting the enzyme from denaturing factors and contaminating proteases (14).

We used this technique with neutral endopeptidase from two distinct tissue sources. First, partially purified preparations of enzyme from human kidney or from bovine nuchal ligament were subjected to SDS-polyacrylamide electrophoresis to separate the proteins. Then the gels were divided, so that replicate lanes for each enzyme were either stained for protein or impregnated with the fluorogenic substrate.

Figure 1 shows the proteins obtained from FPLC fractionation of neutral endopeptidase from either kidney or ligament on a mono Q column. Material from either tissue source had a similar pattern, including two bands with Mr around 95,000 and several additional bands of lower molecular weight. Most of the contaminating protein in the applied preparation was associated with material that had an Mr of approximately 50,000 (data not shown). The

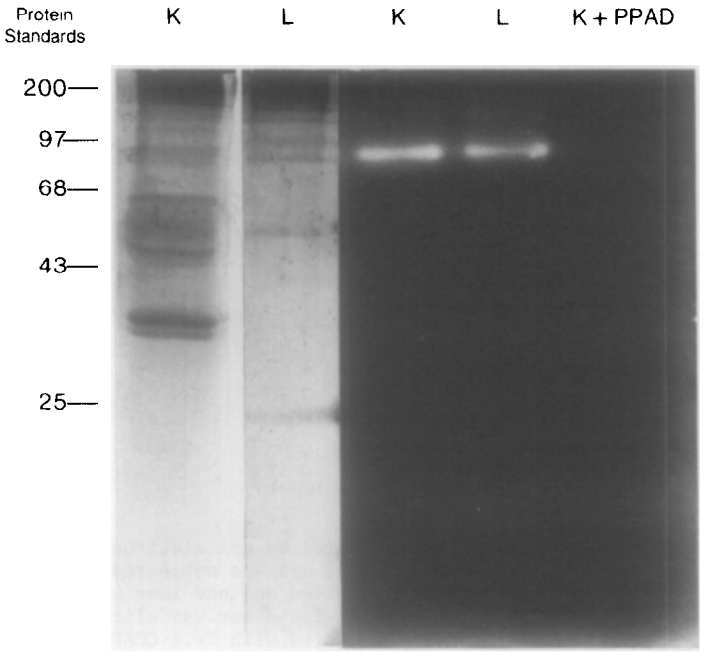

Figure 1. Identification of neutral endopeptidase by fluorescent product formation. Active fractions of neutral endopeptidase from either kidney (K) or ligament (L) membranes were pooled and concentated. Five µg of protein (containing approximately 100 ng of neutral endopeptidase) was applied to a stacking gel (8%) and current of 10 mA was applied to run the sample into the gel. Electrophoresis was at 20 mA at constant voltage. The gels were divided and incubated with substrate alone or in the presence of phophoramidon (PPAD). Mr indicated at the left was estimated from protein standards (Pharmacia) separated in another lane of the gel.

developed fluorescence was limited to a principal band with Mr of approximately 95,000 and a minor band at approximately 200,000 for either preparation. The complete lack of reaction in the gel that was impregnated with substrate in the presence of phosphoramidon (PPAD) suggests the specificity of the reaction for neutral endopeptidase.

In another experiment, partially-purified material from human kidney was separated on two gels run in parallel. One was sliced into 1 mm slices and the proteins were extracted from each slice with Tris-CHAPS buffer. The samples were assayed for neutral endopeptidase using the DAGNPG substrate according to Florentin et al (11). Figure 2 shows that both bands of the 95,000 material are active, but a major protein contaminant (Mr 50,00) had only slight activity. Thus, the substrate gel technique might be a useful preparative procedure when only small amounts of material are available.

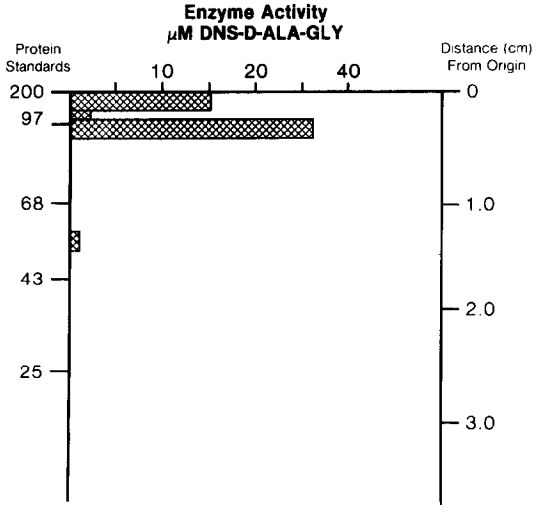

Figure 2. Enzyme activity in bands separated by gel electrophoresis. Partially purified human kidney enzyme (100 ng) was subjected to gel electrophoresis. The gel was divided and one lane was impregnated with the substrate as described. The other was sliced into 2 mm segments. Each segment was extracted with Tris-CHAPS buffer and assayed for enzyme activity using DAPGN as a substrate. The length of each horizontal bar indicates the difference between fluorescence in noninhibited and PPAD-inhibited samples. Position of bands in the gel is indicated on the right axis, and approximate Mr is indicated on the left axis.

Neutral endopeptidase in the substrate-impregnated gels was quantitated by laser densitometry scanning of the photograph negatives. Figure 3 shows the correlation between the amount of enzyme added and the density of the negative obtained following photography of the fluorescent product. The inset shows the corresponding fluorescent bands in the gels where varying amounts of the enzyme were applied. Fluorescence intensity can be measured directly with appropriate instrumentation, but, because it fades within a matter of hours, the gels were photographed to provide a permanent record.

Both human kidney and bovine nuchal ligament enzymes exhibited a similar pattern on standard protein gels. Enzymatic activity was associated with two bands with Mr of approximately 95,000. The similarity in the two preparations is not suprising as cloning studies show that neutral endopeptidase is highly conserved among species (15,16). Other investigators reported an Mr of 90,000 for purified human kidney enzyme (17), 94,000 for rabbit kidney enzyme (18) 94,000 for enzyme from membranes of dermal fibroblasts (19) and 100,000 for enzyme from membranes of human neutrophils (6).

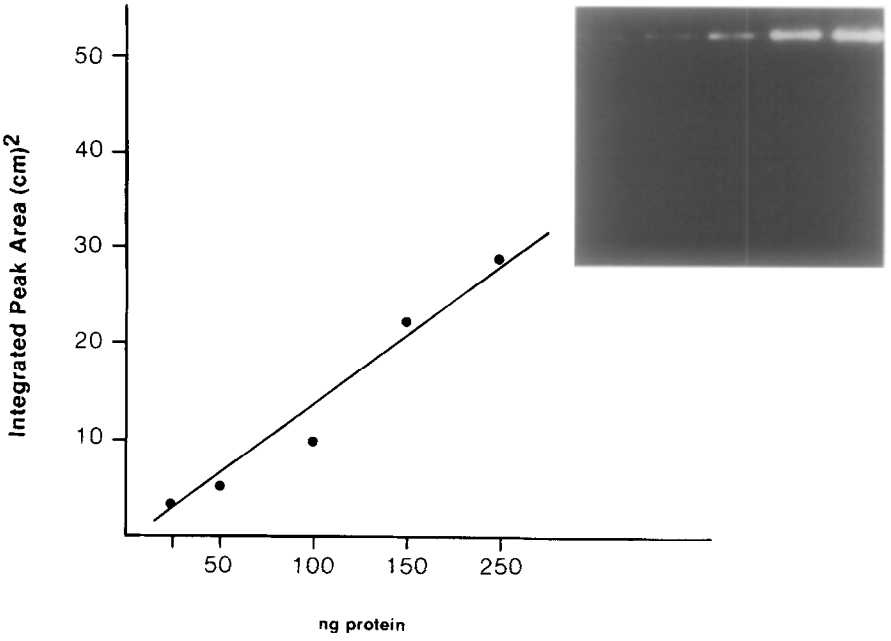

Figure 3. Quantitation of the fluorescent product by scanning densitometry and relation to amount of enzyme applied. Varying amounts of partially purified kidney enzyme were applied to 5 lanes of a standard gel. The proteins were separated by electrophoresis and the gel was impregnated with DAPGN as described in the text. The fluorescent bands that developed after overnight incubation were photographed, and the negatives were scanned with a laser densitometer. The integrated peak values after correction for background were related to the amount of enzyme applied to the gel. The enzyme concentration in each sample was estimated by comparison of specific activities with that of purified neutral endopeptidase. The inset shows the intensity of the fluorescent bands from each enzyme sample applied to the gel. Each band corresponds to a point on the densitometry plot.

We found reaction of immobilized enzyme with the fluorescent substrate at two distinct sites in the gel, and we found that gel slices within the area of 200,000 and 95,000 contained active enzyme. The higher molecular weight band might be a dimeric form, or it might be a distinct higher molecular form of neutral endopeptidase. To this point, when a partially purified preparation from human kidney was separated by FPLC using a superose 12 column, enzyme activity eluted just after the void volume but before an IgG marker (160,000) (data not shown).

The slightly higher Mr for our preparations compared to purified renal neutral endopeptidase may be due to differences in glycosylation patterns. In previous studies we reported that neutral endopeptidase from placenta subjected to SDS-PAGE and electroblotted with antibody to human kidney neutral endopeptidase had immunoreactivity localized in a double band with an Mr

slightly higher than the 90 kD of purified renal enzyme (20). When the placental preparation was treated with neuraminidase prior to electrophoresis it migrated as a single band with a Mr of 90,000. Our present data are consistent with the idea that partially purified neutral endopeptidase from various sources is heterogenous with respect to glycosylation.

Studies of neutral endopeptidase using the substrate-impregnated gels combine the advantages of molecular separation by gel electrophoresis with rapid and easy identification of the active molecular species. It does not require completely purified material as the active enzyme is first separated from contaminating proteins by elecrophoresis. The enzymatic activity can be then quantitated using laser densitometry scanning. This technique should prove useful for both isolation and identification of neutral endopeptidase in small amounts of material, such as that in membrane fractions or cultured cells.

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REFERENCES

1. Erdos, E.G. and Skidgel, R.A. (1989) FASEB J. 3, 145-151.

2. Turner, A.J. (1987) in Neuropeptides and their peptidases. A.J. Turner, ed., Ellis-Horwood, Chichester, 183-201.

3. Bowes, M.A. and Kenny, A.J. (1986) Biochem. J. 236, 801-810.

4. Johnson, A.J., Ashton, J., Schulz, W.W. and Erdos, E.G. (1985) Am. Rev. Resp. Dis. 132, 564-568.

5. Connelly, JC, Skidgel, RA, Schulz, WW, Johnson, AR., Erdos, EG (1985) Proc. Natl. Acad. Sci. USA 82, 8737-8741.

6. Painter, R.G., Dukes, R., Sullivan, J., Carter, R., Erdos, E.G. and Johnson, A.R. (1988) J. Biol. Chem. 263, 9456-9461.

7. Letarte, M., Vers, S., Tran, R., Addis, J.B.L., Onizuka, R.J., Quackenbush, E.J. Joneneel, C.B. andMcInnes, R.R. (1988) J. Exp. Med. 168, 1247-1253, 1988.

8. Erdos, E.G., Schulz, W.W., Gafford, J.T. and Defendini, R. (1985) Lab. Invest. 52, 437-447, 1985.

9. Schulz, W.W., Hagler, H.K., Buja, M. and Erdos, E.G. (1988) Lab. Invest. 59, 789-797.

10. Laemmli, U.K. (1970) Nature 227, 680-685.

11. Florentin, D., Sassi, A. and Roques, B.P.(1984) Anal. Biochem. 141, 62-69.

12. Bradford, M.M. (1976) Anal. Biochm. 72, 248-254.

13. Banda, M.J., Werb, Z and McKerrow, J.H. (1987) Methods Enzymol. 144, 288-305.

14. Mousan, P. and Combes, D. (1988) Methods Enzymol. 157, 584-598.

15. Malfroy, B., Schofield, P.R.,Kuang, W-J., Seeburg, P.H., Mason, A.J. and Henzel, W.J. (1987) Biochem. Biophys. Res. Comm. 144, 59-66.

16. Devault, A., Lazure, C., Nault, C., Le Moual, H., Seidah, N.G., Chretien, M., Kahn, P., Powell, J., Mallet, J., Beaumont, A., Roques, B.P., Crine, P. and Boileau, G. (1987) EMBO J. 6, 1317-1322.

17. Gafford, J., Skidgel, R.A., Erdos, E.G. and Hersh, L.B. (1983) Biochemistry 22, 3265-3271.

18. Crine, P., Legrimellec, C., Lemieux, E., Labonte, L., Fortin, S., Blanchier, A. and Aubry, M. (1985) Biochem. Biophys. Res. Comm. 131, 255-261.

19. Lorkowski, G., Zijderhand-Bleekemolen, J.E., Erdos, E.G., Von Figura, K. and Hasilik, A. (1987) Biochem. J. 248, 345-350.

20. Johnson, A.R., Skidgel, R.A., Gaffort, J.T. and Erdos, E.G. (1984) Peptides 5,789-796, 1984.