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A high-molecular-mass neutral endopeptidase-24.5 from human lung

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A high-M_r neutral endopeptidase-24.5 (NE) that cleaved bradykinin at the Phe[5]–Ser[6] bond was purified to apparent homogeneity from human lung by $(NH_4)_2SO_4$ fractionation, ion-exchange chromatography and gel filtration. The final enzyme preparation produced a single enzymically active protein band after electrophoresis on a 5% polyacrylamide gel. Human lung NE had an M_r of 650000 under non-denaturing conditions, but after denaturation and electrophoresis on an SDS/polyacrylamide gel NE dissociated into several lower-M_r components (M_r 21000–32000) and into two minor components (M_r approx. 66000). The enzyme activity was routinely assayed with the artificial substrate Z-Gly-Gly-Leu-Nan (where Z- and -Nan represent benzyloxycarbonyl- and *p*-nitroanilide respectively). NE activity was enhanced slightly by reducing agents, greatly diminished by thiol-group inhibitors and unchanged by serine-proteinase inhibitors. Human lung NE was inhibited by the univalent cations Na^+ and K^+. No metal ions were essential for activity, but the heavy-metal ions Cu^{2+}, Hg^{2+} and Zn^{2+} were potent inhibitors. With the substrate Z-Gly-Gly-Leu-Nan a broad pH optimum from pH 7.0 to pH 7.6 was observed, and a Michaelis constant value of 1.0 mM was obtained. When Z-Gly-Gly-Leu-Nap (where -Nap represents 2-naphthylamide) was substituted for the above substrate, no NE-catalysed hydrolysis occurred, but Z-Leu-Leu-Glu-Nap was readily hydrolysed by NE. In addition, NE hydrolysed Z-Gly-Gly-Arg-Nap rapidly, but at pH 9.8 rather than in the neutral range. Although human lung NE was stimulated by SDS, the extent of stimulation was not appreciable as compared with the extent of SDS stimulation of NE from other sources.

INTRODUCTION

Endopeptidases have a major role in the hydrolysis of peptide bonds that spans the range from the cleavage of dietary protein to the cleavage of highly potent biologically active peptides and their immediate precursors (Neurath, 1985). The latter role, an aspect of the post-translational processing of peptides, includes the cleavage of bradykinin (BK), other vasoactive peptides and their precursors, e.g. high-M_r kininogen. BK is cleaved by aminopeptidase P (EC 3.4.11.9), by prolyl endopeptidase (EC 3.4.21.26), by dipeptidyl carboxypeptidase (EC 3.4.15.1) (also known as kininase II or angiotensin-converting enzyme), by arginine carboxypeptidase (EC 3.4.17.3) (also known as carboxypeptidase N) and by several endopeptidases. One of these endopeptidases has been designated neutral endopeptidase (NE) (EC 3.4.24.5); it cleaves the Phe[5]–Ser[6] bond of BK.

There have been reports on NE from animal tissues (Camargo *et al.*, 1973; Akopyan *et al.*, 1979; Horsthemke & Bauer, 1980; Wilk & Orlowski, 1980; Ray & Harris, 1985), and a few reports of limited scope on NE from human tissues. Wilk & Orlowski (1980, 1983) purified a high-M_r NE (M_r approx. 700000) from bovine pituitary, which possesses a broad specificity towards BK and other biologically active peptides as well as synthetic chromogenic peptides. By using the latter substrates these authors have postulated that this high-M_r NE is a complex peptidase and has multi-catalytic properties. It possesses three distinct activities, a chymotrypsin-like activity, cleaving bonds on the carboxy side of hydrophobic amino acids, a trypsin-like activity, cleaving bonds in which the carbonyl group of arginine is involved, and an activity that cleaves bonds on the carboxy side of glutamic acid residues. However, this enzyme does not hydrolyse the classical substrates for either trypsin or chymotrypsin. More recently, two high-M_r NEs, which are believed to be similar to, if not identical with, that of bovine pituitary, have been isolated from bovine lens (Ray & Harris, 1985) and rat skeletal muscle (Dahlmann *et al.*, 1985*a*). These enzymes hydrolyse not only small peptides but also some proteins such as crystallins and casein. Since NE has been shown to catalyse the hydrolysis of the Phe[5]–Ser[6] bond of BK, it is of interest to us as a potentially functional kininase, in the physiological sense. In particular, we have focused on lung because the endothelium of lung is critically important for the metabolism of vasoactive peptides (Porter & Whelan, 1980). One of our goals is to determine the relative contribution of each of the BK-cleaving enzymes mentioned above to the overall catabolism of BK by lung. As a part of this endeavour, we have isolated and characterized aminopeptidase P from bovine lung (Szechinski *et al.*, 1983) and human lung (Sidorowicz *et al.*, 1984) and also dipeptidyl

Abbreviations used: NE, neutral endopeptidase-24.5; BK, bradykinin; Z-, benzyloxycarbonyl-; Bz-, benzoyl-; Suc-, 3-carboxypropionyl-; -Nap, 2-naphthylamide; -Nan, *p*-nitroanilide; Tos-, toluene-*p*-sulphonyl.

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peptidase IV (Běhal *et al.*, 1983) and prolyl endopeptidase from human lung (Zolfaghari *et al.*, 1986). We now report on the purification and characterization of human lung NE.

EXPERIMENTAL

Materials

Human lung was obtained at autopsy, only from cases where there was no evidence of pulmonary disease, and only when autopsy was performed or the body was refrigerated within 4 h after death. Lung tissue was stored at −20 °C. DEAE-Bio-Gel A and Bio-Gel A 1.5m were from Bio-Rad Laboratories, Richmond, CA, U.S.A. The substrates Z-Gly-Gly-Leu-Nan, Z-Gly-Gly-Leu-Nap, Z-Gly-Gly-Phe-Nap and BK fragments were purchased from Bachem Feinchemikalien, Bubendorf, Switzerland. Agarose, Tos-Lys-CH_2Cl, Tos-Phe-CH_2Cl, Z-Gly-Gly-Arg-Nap, Z-Arg-Nan, Z-Phe-Nan, Z-Leu-Nap, Bz-DL-Arg-Nan and Suc-Phe-Nan were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Glutaryl-Gly-Gly-Phe-Nap was obtained from Serva Fine Biochemicals, Westburg, NY, U.S.A. The substrate Z-Leu-Leu-Glu-Nap was kindly given by Dr. S. Wilk, Mount Sinai School of Medicine, New York, NY, U.S.A. T.l.c. Redi-Plates Silica Gel-G (20 cm × 20 cm) were purchased from Fisher Scientific Co., Pittsburg, PA, U.S.A. Captopril was from E. R. Squibb and Sons, Princeton, NJ, U.S.A.

NE activity assay

NE activity was assayed with the substrate Z-Gly-Gly-Leu-Nan by the method of Wilk & Orlowski (1979). The reaction mixture contained 0.05 ml of substrate (5 mM in dimethyl sulphoxide), 0.85 ml of 50 mM-Tris/HCl/0.5 mM-EDTA buffer, pH 7.5, and 0.10 ml of enzyme solution. After incubation at 37 °C for 30 min, the enzyme reaction was stopped by the addition of 0.5 ml of 40% (w/v) trichloroacetic acid. NE activity was determined by measuring the rate of release of *p*-nitroaniline by the method of Bratton & Marshall (1939) as modified by Goldbarg & Rutenburg (1958), with the absorbance being measured at 546 nm. For those substrates with 2-naphthylamide rather than *p*-nitroanilide, the same colorimetric procedure was followed except that the absorbance was measured at 580 nm. The unit of neutral endopeptidase activity was defined as that amount of NE required to liberate 1 μmol of *p*-nitroaniline/h at 37 °C under these assay conditions. For the determination of reaction velocities for the estimation of the Michaelis constant, the rate of liberation of *p*-nitroaniline was determined directly at 390 nm.

To confirm the site of BK cleavage by NE, 0.625 μmol of BK was dissolved in 0.4 ml of 10 mM-Tris/HCl/0.5 mM-EDTA buffer, pH 7.5; then 0.1 ml of NE solution (0.4 unit) was added and the mixture was incubated at 37 °C. Separate incubation mixtures were prepared for various time intervals up to 22 h. At the various selected time intervals, incubation mixtures were put in a boiling-water bath to stop the reaction, after which the mixtures were centrifuged at 10000 *g* for 30 min. A 25 μl portion of each clarified supernatant was spotted on a t.l.c. plate. The t.l.c. plates were developed in butan-1-ol/water/pyridine/acetic acid (15:12:10:3, by vol.) for 5 h. After drying, the t.l.c. plates were sprayed with ninhydrin (1% in methanol), and then heated at 100 °C for 5–10 min. The R_F value for each spot was determined and compared with R_F values for known peptides.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard; in some cases protein was measured by the u.v.-absorbance method (Kalckar, 1946).

Polyacrylamide-gel electrophoresis

Electrophoresis under non-denaturing conditions was done by the procedure of Davis (1964); gels were stained with Coomassie Brilliant Blue. NE activity was located by cutting the gels into 4 mm segments, after which the individual segments were incubated with substrate solution and buffer for 2 h. Then the liberated *p*-nitroaniline was determined as described above.

For electrophoresis under denaturing conditions, samples were dissociated as described by Wilk & Orlowski (1983), and then electrophoresis in SDS/polyacrylamide gels (12% polyacrylamide gel containing 0.1% SDS) was carried out by the procedure of Laemmli (1970). The M_r marker proteins were lysozyme (M_r 14300), α-lactoglobulin (M_r 18400), trypsinogen (M_r 24000), pepsin (M_r 34700), egg albumin (M_r 45000) and bovine plasma albumin (M_r 66000).

Gel filtration for M_r determination

The M_r of the native enzyme was estimated on a Bio-Gel A 1.5m column (1.5 cm × 100 cm) equilibrated with either 0.1 M-Tris/HCl/0.5 mM-EDTA buffer, pH 7.5, or 0.1 M-NaCl/50 mM-Tris/HCl/0.5 mM-EDTA buffer, pH 7.5. The flow rate of the column was 18 ml/h and 2 ml fractions were collected. The column was calibrated with standards of known M_r; these were thyroglobulin (M_r 669000), apoferritin (M_r 443000), β-amylase (M_r 200000), alcohol dehydrogenase (M_r 150000), albumin (M_r 66000) and carbonic anhydrase (M_r 29000). The procedure was that of Andrews (1964); the void-volume marker was Blue Dextran (M_r 2000000). Both NE activity and protein concentration were measured in each fraction, then V_e for the enzyme was determined.

RESULTS

Purification of human lung neutral endopeptidase-24.5

Frozen lung was thawed and cut into small pieces (about 3–4 g each) and freed from vascular and membranous tissues. A 50 g portion of cut lung was put immediately into 200 ml of 50 mM-Tris/HCl/0.5 mM-EDTA buffer, pH 7.5, and then homogenized with a Sorvall Omni-Mixer homogenizer for six cycles (each cycle consisted of 1 min full speed followed by 2 min stop in order to allow the dissipation of heat) with the homogenizing chamber lowered into a crushed-ice/water bath. The resulting homogenate was clarified by centrifugation at 37000 *g* for 30 min, and a total of 210 ml of supernatant was obtained. The supernatant was slowly adjusted to 40% saturation with $(NH_4)_2SO_4$ with constant mixing under N_2. The precipitate was collected by centrifuging at 37000 *g* for 30 min and then discarded. The supernatant was then adjusted to 70% saturation with $(NH_4)_2SO_4$; the precipitate containing the activity was dissolved in 40 ml of 0.1 M-Tris/HCl/

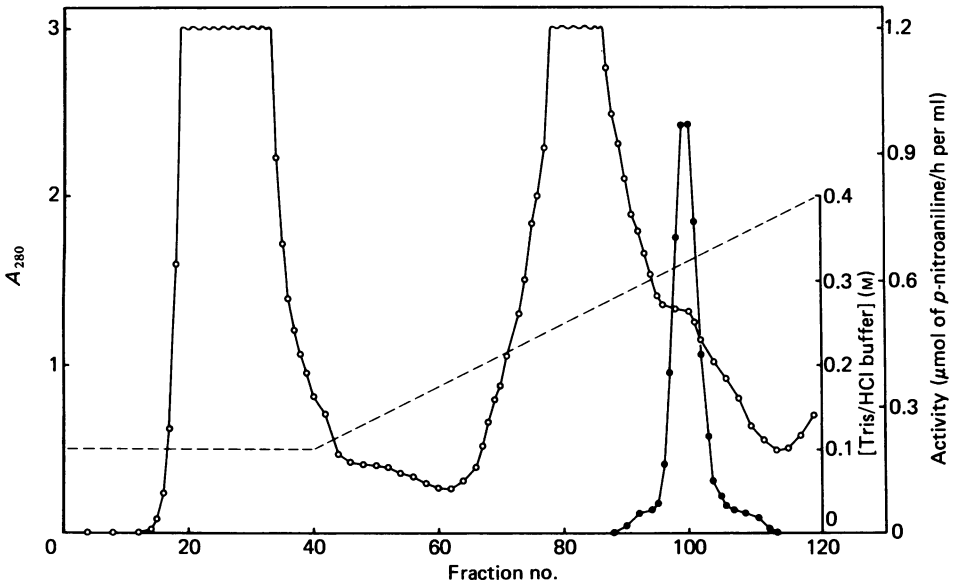

Fig. 1. DEAE-Bio-Gel A chromatography of NE-containing sample obtained from $(NH_4)_2SO_4$ fractionation

The NE-containing precipitate obtained between 40% and 70% saturation with $(NH_4)_2SO_4$ was dissolved in 40 ml of 0.1 M-Tris/HCl/0.5 mM-EDTA buffer, pH 7.5, and then dialysed against this same buffer. Then the sample was applied to a DEA-Bio-Gel A column equilibrated with the same buffer; the NE activity was eluted as described in the text (----, Tris/HCl gradient). ○, A_{280}; ●, NE activity. Fractions with an NE activity greater than 0.088 μmol/h per ml were pooled for further purification.

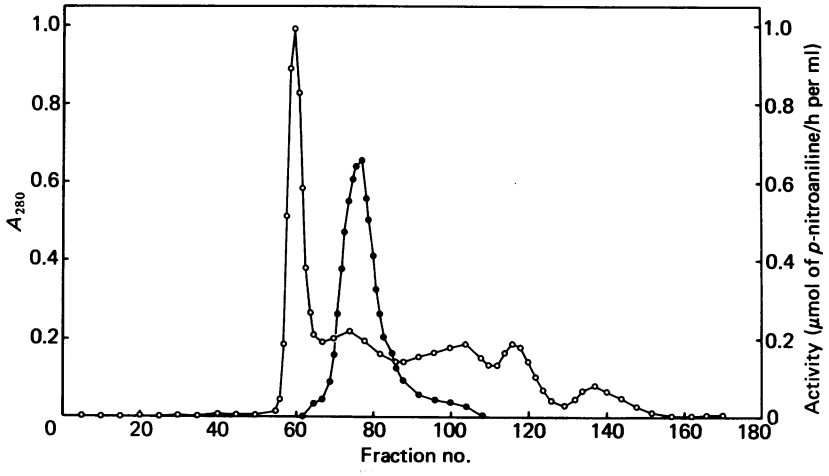

Fig. 2. Gel filtration of NE-containing sample obtained from DEAE-Bio-Gel A chromatography

Pooled and concentrated fractions (final volume 10 ml) from DEAE-Bio-Gel A chromatography were dialysed against 0.1 M-Tris/HCl/0.5 mM-EDTA buffer, pH 7.5. Then the sample was applied to a Bio-Gel A 1.5m column; the void volume was 124 ml; the total volume was 450 ml; the total settled bed volume was 490 ml. ○, A_{280}; ●, NE activity. Fractions with an NE activity greater than 0.11 μmol/h per ml were pooled for further purification.

0.5 mM-EDTA buffer, pH 7.5. The resulting solution was then dialysed three times against 4 litres of the same buffer.

The dialysed solution was applied to a 2.5 cm × 30 cm column containing DEAE-Bio-Gel A that had previously been packed and equilibrated with 0.1 M-Tris/HCl/0.5 mM-EDTA buffer, pH 7.5. The equilibrium buffer was passed over the column at a flow rate of 30 ml/h until 40 fractions each containing 7.5 ml were collected. The bound protein was then eluted by a linear gradient established with 300 ml of equilibration buffer and 300 ml of 0.4 M-Tris/HCl/0.5 mM-EDTA buffer, pH 7.5. All of the fractions were monitored for protein concentration (absorbance at 280 nm method) and for enzymic activity. A single peak of activity emerged from the column near the end of the gradient (Fig. 1). Fractions that catalysed the liberation of more than 0.088 μmol of *p*-nitroaniline/h per ml were pooled and concentrated to 10 ml (Amicon XM-50 membrane).

A 2.5 cm × 100 cm column of Bio-Gel A 1.5m was

Table 1. Purification of human lung NE

During this purification process, the substrate used for NE assay was Z-Gly-Gly-Leu-Nan; the assay procedure is as described in the Experimental section. The unit of activity is defined as that amount of enzyme required to convert 1 μmol of substrate into product/h at 37 °C under the conditions of the assay. Specific activity is defined as units of activity/mg of protein. The data below are the means ± S.E.M. for three preparations starting from 50 g of lung.

Step	Total units (μmol/h)	Recovery (%)	Protein (mg)	Specific activity (μmol/h per mg)	Purification (fold)
Centrifuged homogenate (37000 *g* for 30 min)	39 ± 2.3	100	3077 ± 434	0.0127 ± 0.001	1
$(NH_4)_2SO_4$ fraction (40–70% saturation)	41 ± 4.3	105	1690 ± 329	0.0241 ± 0.005	1.9
DEAE-Bio-Gel A chromatography	32 ± 1.4	82	38 ± 2.5	0.831 ± 0.03	66
Gel filtration on Bio-Gel A 1.5m	20 ± 1.9	52	10 ± 0.9	1.99 ± 0.23	157
DEAE-Bio-Gel A chromatography (second)	15.5 ± 2.1	40	4.6 ± 0.5	3.37 ± 0.18	265

equilibrated with 0.1 M-Tris/HCl/0.5 mM-EDTA buffer, pH 7.5. The concentrated sample was then applied to the column and eluted with the same buffer at a flow rate of 20 ml/h. A total of 170 fractions, 4 ml each, were collected and monitored for protein and enzymic activity as described above. The enzyme was eluted as a single peak, and fractions that catalysed the liberation of more than 0.11 μmol of *p*-nitroaniline/h per ml were pooled (Fig. 2).

As the final purification step, the pooled fractions from above were applied to a 1.5 cm × 30 cm column of DEAE-Bio-Gel A that had been equilibrated with 0.1 M-Tris/HCl/0.5 mM-EDTA buffer, pH 7.5. The flow rate was 21 ml/h, and 3.5 ml fractions were collected. After the first 20 fractions, the column was eluted with a linear gradient established with 175 ml of equilibration buffer and 175 ml of 0.4 M-Tris/HCl/0.5 mM-EDTA buffer, pH 7.5. A total of 117 fractions were obtained, and each fraction was monitored for both protein and activity as described above. One major protein peak emerged from the column, and this coincided with the NE activity. The fractions that catalysed the liberation of more than 0.15 μmol of *p*-nitroaniline/h per ml were pooled and concentrated to 1 mg of protein/ml for further analysis.

At each stage of the purification, samples were taken and assayed for NE activity and for protein content. The results of the purification are shown in Table 1. The purified NE had a final specific activity of 3.4 μmol of *p*-nitroaniline/h per mg of protein, and 40% of the original activity was recovered.

Some properties of purified human lung neutral endopeptidase-24.5

Purified NE was subjected to polyacrylamide-gel electrophoresis, under non-dissociating conditions. A single band of enzymically active protein was observed on both 5% and 7.5% gels (Fig. 3). At the higher gel concentration, migration of the enzyme was greatly retarded, and NE appeared as a single sharp protein band very near the top of the gel. Purified NE was applied to a gel-filtration column (1.5 cm × 100 cm) of Bio-Gel A 1.5 m that had been calibrated with the M_r standards listed in the Experimental section. NE was

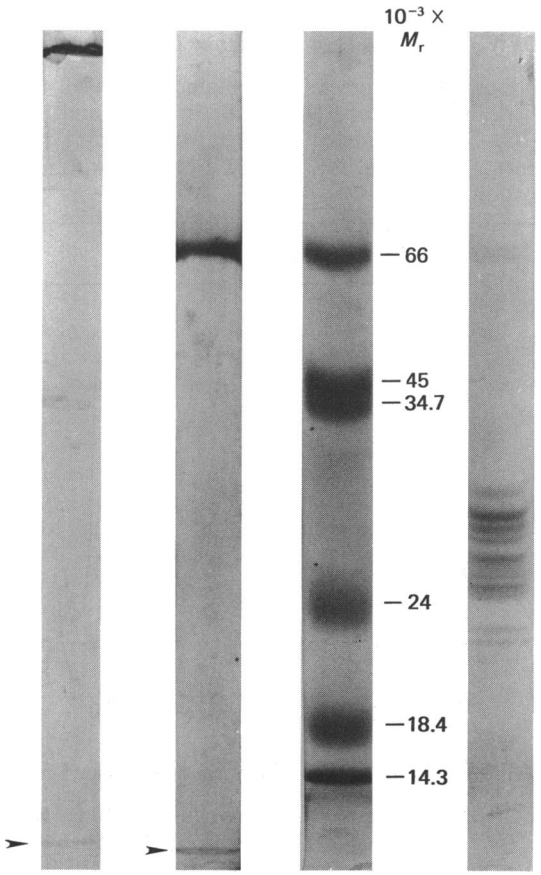

Fig. 3. Polyacrylamide-gel electrophoresis of purified NE

The gels shown are, from left to right, as follows: electrophoresis of purified NE under non-denaturing conditions in a 7.5% gel; electrophoresis of purified NE under non-denaturing conditions in a 5% gel; electrophoresis of M_r standards (from top to bottom: bovine plasma albumin, egg albumin, pepsin, trypsinogen, α-lactoglobulin and lysozyme) in an SDS/polyacrylamide gel; electrophoresis of purified NE in an SDS/polyacrylamide gel. M_r values are shown alongside the SDS/polyacrylamide gel with the standards; arrows indicate the position of tracking dye for the two non-denaturing gels. The composition of the gels and other details are given in the text.

eluted as one sharp peak immediately following thyroglobulin with either 0.1 M-Tris/HCl/0.5 mM-EDTA buffer, pH 7.5, or with 0.1 M-NaCl/50 mM-Tris/HCl/0.5 mM-EDTA buffer, pH 7.5, as the eluting buffer. This indicated an apparent M_r of 650000 for human lung NE. After dissociation and electrophoresis on SDS/polyacrylamide gel as described in the Experimental section, lung NE yielded several low-M_r components (M_r range 21000–32000) and two minor higher-M_r (66000) components (Fig. 3).

A broad pH optimum from pH 7.0 to pH 7.6 was observed with the substrate Z-Gly-Gly-Leu-Nan.

The effects of various inhibitors and metal ions on NE are shown on Tables 2 and 3. Of the inhibitors, only those that bind tightly to thiol groups were effective; neither Tos-Lys-CH_2Cl, Tos-Phe-CH_2Cl, phenylmethanesulphonyl fluoride, Captopril (a potent kininase II inhibitor) nor phosphoramidon (a potent endopeptidase-24.11 inhibitor) inhibited human lung NE. Heavy-metal ions were inhibitory, as were the univalent cations Na^+ and K^+.

Compounds cleaved by purified human lung neutral endopeptidase-24.5

The Michaelis constant was determined for NE with the substrate Z-Gly-Gly-Leu-Nan. The exceedingly low solubility of this compound in water necessitated the use of dimethyl sulphoxide in the preparation of substrate solutions as described in the Experimental section. The rate of hydrolysis of this substrate was proportional to time and to NE concentration during a 3 h incubation period. The velocity of Z-Gly-Gly-Leu-Nan hydrolysis over the concentration range 0.02–0.20 mM at pH 7.5 was determined. The NE concentration was 12.5 μg/ml (19 nM). Duplicate velocity values were determined at each substrate concentration point; the average velocity values were used with the double-reciprocal (Lineweaver–Burk) method and the least-squares linear-regression method to calculate a value for the Michaelis constant. A K_m value of 1.0 mM was obtained; $V_{max.}$ and $k_{cat.}$ values were 0.21 μmol/min per mg of NE and 136/min respectively. The $k_{cat.}/K_m$ value, calculated from the data above, was 136/min per mM. Dixon & Webb (1979) have pointed out the error in $k_{cat.}/K_m$ that occurs as a function of relative substrate concentration; since the mid-point of our substrate concentration range is approx. 10% of K_m, we would expect an error on the order of 9%. When we calculated the $k_{cat.}/K_m$ value, as suggested by Dixon & Webb (1979), for NE from the apparently first-order region of our plots of original velocity versus substrate concentration, a value of 131/min per mM was obtained, which is in close agreement with the value calculated by the double-reciprocal (Lineweaver–Burk) method.

The activity of NE on several other artificial substrates was determined, as shown in Table 4. The data are expressed as relative velocities, as compared with the rate of hydrolysis of Z-Gly-Gly-Leu-Nan; when Z-naphthylamide was substituted for *p*-nitroanilide in this substrate, no hydrolysis occurred. However, Z-Leu-Leu-Glu-Nap and Z-Gly-Gly-Arg-Nap were hydrolysed at a significant rate.

The point at which BK is cleaved by NE was determined by identifying the reaction products resulting from incubation of BK with NE, as described in the Experimental section, for various time intervals up to 22 h. For each time interval, a 25 μl portion of the corresponding incubation mixture was spotted on a t.l.c. plate, after which the plates were developed and sprayed with ninhydrin as described above. Beginning with the

Table 2. Influence of various effectors on human lung NE

The incubation mixture contained 0.1 ml of appropriately diluted enzyme (10 μg of protein), 0.1 ml of appropriately diluted effector solution and 0.8 ml of 50 mM-Tris/HCl buffer, pH 7.5, and was preincubated at 37 °C for 10 min. Then 0.05 ml of substrate solution, prepared as described in the text, was added in order to assay the residual NE activity, as described in the text. The values given are the means for three determinations. In all cases the coefficient of variability was 6% or less.

Compound	Final concn. (mM)	Residual activity (%)
Dithiothreitol	0.1	104
	1.0	129
L-Cysteine	0.1	107
	1.0	118
2-Mercaptoethanol	0.1	101
	1.0	113
1,10-Phenanthroline	0.1	103
	1.0	101
EDTA	0.1	99
	1.0	80
Benzamidine	0.1	106
	1.0	103
Iodoacetic acid	0.1	121
	1.0	98
Phenylmethanesulphonyl fluoride	0.1	108
	1.0	105
5,5-Dithiobis-(2-nitrobenzoic acid)	0.1	89
	1.0	48
N-Ethylmaleimide	0.1	80
	1.0	42
p-Chloromercuribenzoic acid	0.1	0
	1.0	0
p-Hydroxymercuribenzoic acid	0.1	0
	1.0	0
Tos-Lys-CH_2Cl	0.1	101
	1.0	89
Tos-Phe-CH_2Cl	0.1	99
	1.0	88
Captopril	0.1	100
	1.0	103
Phosphoramidon	0.1	101
	1.0	84
Trypsin inhibitor (soya-bean)	0.1 mg/ml	121
	1.0 mg/ml	111
Haemoglobin (human)	0.1 mg/ml	117
	1.0 mg/ml	113
Albumin (bovine)	0.1 mg/ml	112
	1.0 mg/ml	106

Table 3. Effect of metal ions on human lung NE

The incubation mixture contained 0.1 ml of appropriately diluted enzyme (10 μg of protein), 0.1 ml of appropriately diluted metal ion solution and 0.8 ml of 50 mM-Tris/HCl buffer, pH 7.5, and was preincubated at 37 °C for 10 min. Then 0.05 ml of substrate solution, prepared as described in the text, was added in order to assay the residual NE activity, as described in the text. The values given are the means for three determinations. In all cases the coefficient of variability was 5% or less.

Compound	Final concn. (mM)	Residual activity (%)
NaCl	1	80
	10	59
	100	59
KCl	1	80
	10	57
	100	51
KH_2PO_4/K_2HPO_4	50	55
	100	57
$CaCl_2$	0.1	100
	1.0	100
$CoCl_2$	0.1	75
	1.0	44
$CuCl_2$	0.1	16
	1.0	1
$FeCl_2$	0.1	43
	1.0	30
$FeCl_3$	0.1	53
	1.0	29
$HgCl_2$	0.1	0
	1.0	0
$MgCl_2$	0.1	99
	1.0	93
$MnCl_2$	0.1	100
	1.0	92
$NiCl_2$	0.1	100
	1.0	48
$SnCl_2$	0.1	109
	1.0	40
$ZnCl_2$	0.1	105
	1.0	3

0.5 h incubation period, two spots other than BK were detected after the ninhydrin application. These two spots were Arg-Pro-Pro-Gly-Phe (R_F 0.59) and Ser-Pro-Phe-Arg (R_F 0.48). At progressively longer incubation times, the size and intensity of these two spots increased, and the size and intensity of the BK spot decreased.

Effect of SDS on purified human lung neutral endopeptidase-24.5

NE was preincubated for 10 min at 37 °C with SDS at various concentrations up to 0.020% at neutral pH (50 mM-Tris/HCl buffer, pH 7.5); then the remaining NE activity was determined. With the substrate Z-Gly-Gly-Leu-Nan the maximum activity enhancement (86%) occurred at 0.002% SDS, and with the substrate Z-Leu-Leu-Glu-Nap the maximum activity enhancement (190%) occurred at 0.004% SDS. Then the influence of SDS was determined with the substrate Z-Gly-Gly-Arg-Nap at alkaline pH {50 mM-Caps [3-(cyclohexylamino)propane-1-sulphonic acid]/NaOH buffer, pH 10.0}. No stimulation was observed, but rather an increasing degree of inhibition occurred, beginning at 0.004% SDS (20% inhibition) and with 60% inhibition at 0.008% SDS.

Table 4. Hydrolysis of synthetic substrates by purified human lung NE

For NE activity measurements, 0.1 ml enzyme solution (10 μg of protein), 0.85 ml of 50 mM buffer (including 0.5 mM-EDTA) and 0.05 ml of substrate solution (prepared as described in the Experimental section) were combined, mixed and incubated for 30 min at 37 °C, after which the rates of release of *p*-nitroaniline or 2-naphthylamine were measured. The buffer was Tris/HCl, pH 7.5, for the first number in each pair of values given; for the second number the buffer was Caps/NaOH, pH 10.0. The results are reported as relative rates; the rate of hydrolysis of the substrate Z-Gly-Gly-Leu-Nan with the Tris/HCl buffer was assigned the value 100. All other rates of hydrolysis are reported relative to the rate for Z-Gly-Gly-Leu-Nan. The values are the means for three determinations; in all cases the coefficient of variability was 4% or less.

Substrate	Relative activity
Z-Gly-Gly-Leu-Nan	100; 62
Z-Gly-Gly-Leu-Nap	0; 0
Z-Leu-Leu-Glu-Nap	113; 3
Z-Gly-Gly-Arg-Nap	6; 56
Glutaryl-Gly-Gly-Phe-Nap	4; 0
Z-Gly-Gly-Phe-Nap	0; 0
Z-Leu-Nap	0; 0
Bz-DL-Arg-Nan	0; 0
Suc-Phe-Nan	0; 0
Z-Arg-Nan	0; 0
Z-Phe-Nan	0; 0

DISCUSSION

Human lung neutral endopeptidase-24.5, when purified to apparent homogeneity as judged by the appearance of a single protein band with NE activity on polyacrylamide-gel electrophoresis, had an M_r value of 650000, which is in good agreement with the NE M_r values reported by other workers for rat skeletal muscle, bovine pituitary gland and bovine lens (Dahlmann *et al.*, 1985*a*; Wilk & Orlowski, 1983; Ray & Harris, 1985). Similarly, the number of components, and their M_r values (21000–32000), yielded by human lung NE on denaturation are consistent with the results also reported by the workers cited above. Lung NE is strongly inhibited by reagents that react with thiol groups, but is not inhibited by either chelating agents or serine-proteinase inhibitors (Table 2). Furthermore, NE is strongly inhibited by Hg^{2+} and other heavy-metal ions, and is also inhibited by the univalent cations Na^+ and K^+, as shown in Table 3. Thus human lung NE is a thiol-group-dependent proteinase that lacks classical trypsin and chymotrypsin activity, as well as dipeptidyl

carboxypeptidase (kininase II) and endopeptidase-24.11 (enkephalinase) activities, as indicated by insensitivity to Captopril and phosphoramidon.

Purified human lung NE hydrolyses BK at the Phe[5]–Ser[6] bond and cleaves several artificial substrates; one of these, Z-Gly-Gly-Leu-Nan, has an estimated K_m value of 1.0 mM. Other workers have speculated about a possible multi-catalytic nature for NE, and some of our data provide particular support for that hypothesis; specifically our data for the substrates Z-Gly-Gly-Leu-Nan and Z-Gly-Gly-Leu-Nap suggest the existence of a catalytic site where the nature of the amino-donating residue of the cleaved bond is one factor influencing substrate reactivity. In addition, our data for these two substrates and for Z-Leu-Leu-Glu-Nap suggest the existence of another site where the substitution of 2-naphthylamide for *p*-nitroanilide is not a determining factor for substrate reactivity. The data for the substrate Z-Gly-Gly-Arg-Nap indicate that a positive charge on the carboxy-donating residue of the cleaved bond is a factor blocking substrate reactivity (Table 4, Z-Gly-Gly-Arg-Nap has low reactivity at neutral pH and higher reactivity at alkaline pH); this result may indicate a third catalytic site. Further speculations on the multi-catalytic nature of NE will be dependent, in part, on the availability of several more artificial substrates.

Although other workers (Wilk & Orlowski, 1983; Dahlmann *et al.*, 1985*b*) have reported significant stimulation (10–14-fold) of NE by SDS, NE from human lung was not stimulated appreciably in comparison with other NEs. The activity of lung NE, as determined with Z-Gly-Gly-Arg-Nap, was not stimulated at all, but rather inhibition resulted, which increased with increasing SDS concentration. This may also indicate a catalytic site that differs from the one(s) susceptible to SDS stimulation.

The occurrence of NE in human lung, along with our earlier reports on human lung aminopeptidase P, dipeptidyl peptidase and prolyl endopeptidase (Sidorowicz *et al.*, 1984; Běhal *et al.*, 1983; Zolfaghari *et al.*, 1986), broadens the range of proteinases known to be in lung that may participate in kinin catabolism; these enzyme studies are part of our overall goal, which is to assess the relative contribution of each of the BK-cleaving enzymes in lung to the total kinin catabolism in lung.

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