5 Sofer, W., and Ursprung, H., J. biol. Chem. *243* (1968) 3110.
6 Baudhuin, P., Beaufay, H., Rahman-Li, Y., Sellinger, O.Z., Wattiaux, R., Jacques, P., and de Duve, C., Biochem. J. *92* (1964) 179.
7 Courtright, J.B., Genetics *57* (1967) 25.
8 Crow, K.E., Kitson, T.M., Mac Gibbon, A.K.H., and Batt, R.D., Biochim. biophys. Acta *350* (1974) 121.
9 Eckfeldt, J.H., Mope, L., Takio, K., and Yonetoni, T., J. biol. Chem. *251* (1976) 236.
10 Schnaitman, C., Erwin, V.G., and Greenawalt, J.W., J. Cell Biol. *32* (1967) 719.
11 Ochoa, S., in: Methods in Enzymology, vol. 1, p. 735. Eds S.P. Colowick and N.O. Kaplan, Academic Press, New York 1955.
12 Vaes, G., in: Methods in Enzymology, vol. 8, p. 509. Eds S.P. Colowick and N.O. Kaplan, Academic Press, New York 1966.
13 Beaufay, H., Amar-Costresec, A., Feytmans, E., Thinès-Sempoux, D., Wibo, M., Robbi, M., and Berthet, J., J. Cell Biol. *61* (1974) 188.
14 Kaidanov, L.Z., Genetica *52/53* (1980) 165.
15 Deltombe-Liétaert, M.C., Delcour, J., Lenelle-Monfort, N., and Elens, A., Experientia *35* (1979) 579.
16 Liétaert, M.C., Libion-Mannaert, M., and Elens, A., Experientia *37* (1981) 689.
17 Hougouto, N., Liétaert, M.C., Libion-Mannaert, M., Feytmans, E., and Elens, A., Genetica *58* (1982) 121.
18 Hougouto, N., Glotov, N.V., and Kaidanov, L.Z., Genetika (Russ.) *16* (1980) 1228.
19 Amosova, I.S., Bugaeva, E.A., and Kaidanov, L.Z., Citol. Genet. (Ukr.) *5* (1983) 49.
20 Dickinson, W.J., and Sullivan, D.T., Gene-enzymes systems in *Drosophila*, Springer, Heidelberg 1975.
21 Garcin, F., Coté, J., Radouco-Thomas, R., Chawla, S., and Radouco-Thomas, C., Experientia *39* (1983) 1122.
22 Eckfeldt, J.H., and Yonetani, T., Archs Biochem. Biophys. *175* (1976) 712.
23 Eriksson, C.J.P., Marselos, M., and Koivula, T., Biochem. J. *152* (1975) 709.
24 Holmes, R.S., in: Alcohol and aldehyde metabolizing systems, vol. 4, p. 57. Ed. R. Thurman. Plenum Press, New York 1980.
25 David, J., Bocquet, C., Arens, M.F., and Fouillet, P., Biochem. Genet. *14* (1976) 989.
26 Dawson, A.G., Trends Biochem. Sci. *8* (1983) 195.
27 Feytmans, E., and Leighton, F., Biochem. Pharmac. *22* (1973) 349.
28 Lieber, C.S., Scient. Am. *234/3* (1976) 25.
29 Teschke, R., Matsuzaki, S., Onishi, K., De Carli, L.M., and Lieber, C.S., Clin. exp. Res. Alcoholism *1* (1977) 7.
30 Havre, P., Abrams, M.A., Corrall, R.J.M., Yu, L.C., Szczepanik, P.A., Feldman, H.B., Klein, P., Kong, M.S., Margolis, J.M., and Landau, B.R., Archs Biochem. Biophys. *182* (1977) 14.

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## Elastase digested urokinase (ED-UK)

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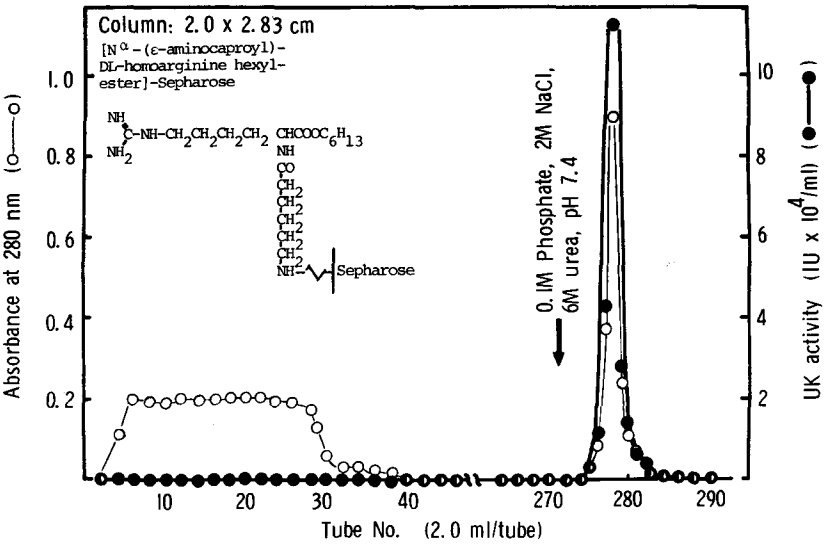
*Summary.* Elastase digested urokinase (ED-UK) was prepared from human high mol. wt urokinase (HMW-UK). It resembled low mol. wt urokinase (LMW-UK) in its mol. wt, specific activity, and active sites. The steady-state kinetic parameters of each enzyme for the activation of human Glu-plasminogen also resembled each other, as did their amidase parameters (with pyro-Glu-Gly-Arg-pNA).
*Key words.* Urokinase; elastase; enzymatic degradation.

Urokinase (EC 3.4.99.26), a serine protease produced in the kidney and found in the urine, is a potent activator of plasminogen and has been employed as a thrombolytic agent[1,2]. Two major molecular forms occur in current therapeutic preparations: a high mol. wt form (HMW-UK), and a low mol. wt form (LMW-UK)[2,3]. Both enzymes consist of two chains[4-8], designated as the B chain and A or $A_1$ chains[9,10], respectively, which are connected by a single interchain disulfide bond[7,8]. Since transformation of HMW-UK to LMW-UK readily occurs in solutions without protease inhibitors[4,11], or by enzyme treatments[12-14], LMW-UK is thought to represent an enzymatic degradation product of native HMW-UK. However, the detailed mechanism of degradation and how the related enzymes really act are not yet well understood. In this study, we succeeded in preparing and characterizing a new fibrinolytic enzyme, ED-UK, derived from HMW-UK.

*Materials and methods.* The following substances were used: hog pancreas elastase (type II) from Sigma Chemical Co., USA; pyro-Glu-Gly-Arg-pNA (S-2444) and H-D-Val-Leu-Lys-pNA (S-2251) from Kabi Group, Inc., USA; and HMW-UK and LMW-UK, purified by affinity chromatography and gel filtration as reported previously[7,15]. UK activity was determined by assessing its ability to hydrolyze pyro-Glu-Gly-Arg-pNA[16]. The enzyme activity was expressed in international units (IU) with Japanese Standard Urokinase (MW 003) as a standard. Active site titration with p-nitrophenyl-p'-guanidinobenzoate (NPGB) was carried out as described by Chase and Shaw[17]. Steady-state kinetic parameters of activation of Glu-plasminogen were determined in a coupled assay in which the plasmin formed was monitored by hydrolysis of H-D-Val-Leu-Lys-pNA at pH 7.4 and 37 °C, as described previously by Wohl et al.[18]. The preparation of [$N^{\alpha}$-($\varepsilon$-aminocaproyl)-DL-homoarginine hexylester]-Sepharose and other procedures of affinity chromatography were as described previously[15]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 10% gels by the method of Weber and Osborn[19]. Isoelectric focussing was performed according to the method of Vesterberg and Svensson[20] using ampholytes of pH 3.5–10.5. Determinations of amino acid composition were performed by the technique of Gros and Labouesse[21]. Immunological methods with rabbit anti HMW-UK and anti human kidney HMW-UK sera were carried out as described previously[22].

*Results and discussion.* 5.50 mg of highly purified HMW-UK (spec. act. = 106,700 IU/mg protein, 92% active) was dissolved in 0.5 ml of 0.05 M phosphate buffer (pH 7.49) containing 0.15 M NaCl and 1.5 µg of hog pancreas elastase, and incubated for 30 min at 25 °C. Under these conditions, the HMW-UK molecule was digested and produced a new enzyme, ED-UK, with approximately 83% of the activity of the intact enzyme still being retained. By SDS-PAGE under nonreducing conditions, the material showed two main protein bands with molecular weights of approximately 31,000 and 19,000. The former had enzyme activity and was named ED-UK, whereas the latter had no activity. When the reactant was reduced with 0.1 M $\beta$-mercaptoethanol, the ED-UK band migrated to a position showing that it had a slightly lower mol. wt of about 30,000, and dis-




Affinity chromatography of ED-UK on [$N^{\alpha}$-(ε-aminocaproyl)-DL-homoarginine hexylester]-Sepharose. 5.50 mg of elastase-digested HMW-UK dissolved in 0.1 M phosphate buffer (pH 7.4) containing 2.0 M NaCl was applied to a [$N^{\alpha}$-(ε-aminocaproyl)-DL-homoarginine hexylester]-Sepharose column. The flow rate was 29 ml/h. The arrow indicates the point of buffer change.

played no activity. On isoelectric focussing, HMW-UK revealed only one activity peak (pI = 9.8), whereas ED-UK included at least five molecular forms with pIs ranging from pH 5.2 to 9.3. The figure shows an affinity chromatogram of such enzymatically digested urokinase (485,000 IU; 5.50 mg protein) on a column of [$N^{\alpha}$-(ε-aminocaproyl)-DL-homoarginine hexylester]-Sepharose. Most of the inactive protein was eluted in the void volume fraction, whereas the ED-UK was adsorbed and could be eluted with phosphate-NaCl buffer (pH 7.4) containing 6 M urea. The active fraction was further purified by Sephadex G-100 gel filtration (1.0 × 150 cm column; 0.1 M phosphate containing 0.2 NaCl and 0.2 M urea). The final product recovered in the main activity peak (yield 239,500 IU; 1.26 mg protein) was found to be homogeneous on SDS-PAGE and yielded a single component on double immunodiffusion with specific urokinase antiserum. Rabbit anti HMW-UK, anti LMW-UK, and also anti human kidney tissue-culture urokinase reacted equally with this antiserum.

The properties of the final preparation are summarized in the table, together with those of the intact enzymes, HMW-UK and LMW-UK. The mol. wt of ED-UK was 31,000–32,000 and its spec. act. was 190,000 IU/mg protein, both of which were similar to those of LMW-UK. Based on NPGB titration, the purified ED-UK was found to be approximately 86% active, and the calculated molar activity was $6.94 \times 10^{-9}$ IU/mmole active site. The amino acid compositions of ED-UK and, for comparison, LMW-UK were determined after hydrolysis for 20 h in 6 N HCl at 110 °C. The values, calculated on the basis of the number of residues per mol of protein, also resembled each other.

The steady-state kinetic parameters for Glu-plasminogen activation and S-2444 amidolysis were also investigated. In the case of Glu-plasminogen activation, the values of the apparent Michaelis constant ($K_m$) for ED-UK and LMW-UK (2.21–2.24 μM) were higher than that of intact HMW-UK (0.63 μM), and the values of the catalytic constant ($K_{cat}$) for ED-UK and LMW-UK (44.1–47.1 $min^{-1}$) were higher than that of HMW-UK (28.1 $min^{-1}$). The values of the overall second-order rate constant, $k_{cat}/K_m$, calculated for ED-UK and LMW-UK were 19.3–20.0 $\mu M^{-1}min^{-1}$, which was lower than that of HMW-UK (44.6 $\mu M^{-1}min^{-1}$). On the other hand, the amidase parameters of ED-UK, LMW-UK and HMW-UK were all similar, with $k_{cat}/K_m$ values of 0.26, 0.26 and 0.34 $\mu M^{-1}min^{-1}$, respectively.

Based on these results, ED-UK appears to resemble LMW-UK in both its physical and chemical properties and in its basic enzymatic functions. Recently, we have also reported the enzymatic degradation of urinary trypsin-plasmin inhibitor[23,24], plasmin and human kidney tissue cultured single-chain HMW-UK[14] to lower molecular forms. The detailed properties and physiological significance of such enzymatically modified fibrinolytic components remain to be elucidated.

Comparison of physicochemical properties

| | HMW-UK | ED-UK | LMW-UK |
|---|---|---|---|
| Molecular weight | | | |
| Gel filtration | 53,800 | 31,500 | 33,200 |
| SDS-PAGE | 53,000 | 31,000 | 31,500 |
| Spec. act. (IU/mg protein) | 124,000 | 190,000 | 230,000 |
| Active site % (NPGB) | 96.5 | 86.3 | 91.2 |

Amino acid composition of ED-UK (Trp not determined; values not corrected). The values in parentheses are those of LMW-UK* for comparison.

| | | | | | |
|---|---|---|---|---|---|
| Lys | 18.0 | (18.0) | Ala | 12.1 | (12.7) |
| His | 9.2 | (9.3) | 1/2 Cys | 10.0 | (10.1) |
| Arg | 14.0 | (13.9) | Val | 12.2 | (12.6) |
| Asp | 22.5 | (22.6) | Met | 4.5 | (4.8) |
| Thr | 20.1 | (20.7) | Ile | 14.5 | (15.7) |
| Se | 21.0 | (20.6) | Leu | 21.2 | (22.0) |
| Glu | 29.5 | (30.1) | Tyr | 12.5 | (12.5) |
| Pro | 17.0 | (16.4) | Phe | 9.0 | (9.7) |
| Gly | 30.2 | (27.3) | | | |

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1 Urokinase Pulmonary Embolism Trial Study Group, J. Am. med. Ass. *229* (1974) 1606.
2 Ong, E.B., Soberano, M.E., Johnson, A.J., and Schoellman, G., Thromb. Haemostas. *38* (1977) 801.
3 Soberano, M.E., Ong, E.B., Johnson, A.J., Levy, M., and Schoellmann, G., Biochim. biophys. Acta *445* (1976) 763.
4 Nobuhara, M., Sakamaki, M., Onishi, H., and Suzuki, Y., J. Biochem. *90* (1981) 225.
5 Gunzler, W.A., Steffens, G.J., Otting, F., Buse, G., and Flohe, L., Hoppe-Seylers Z. physiol. Chem. *363* (1982) 133.
6 Schaller, J., Nick, H., Rickli, E.E., Gillessen, D., Lergier, W., and Studer R.O., Eur. J. Biochem. *125* (1982) 251.
7 Sumi, H., and Robbins, K.C., J. biol. Chem. *258* (1983) 8014.
8 Miwa, N., Obata, Y., and Suzuki, A., Biochem. biophys. Res. Commun. *112* (1983) 754.
9 Gunzler, W.A., Steffens, G.J., Otting, F., Kim, S.M.A., Franskus, E., and Flohe, L., Hoppe-Seylers Z. physiol. Chem. *363* (1982) 1153.

10 Steffens, G.J., Gunzler, W.A., Otting, F., Frankus, E., and Flohe, L., Hoppe-Seylers Z. physiol. Chem. *363* (1982) 1043.
11 Johnson, A.J., Soberano, M., Ong, E.B., Levy M., and Schoellmann, G., in: Thrombosis and Urokinase, p. 59. Ed. R. Paeletti and S. Sherry. Academic Press, New York 1977.
12 Lesuk, A., Terminiello, L., Traver, J.H., and Groff, J.L., Fedn Proc. *26* (1967) 647.
13 Barlow, G.H., Francis, C.W., and Marder, V.J., Thromb. Res. *23* 541 (1981).
14 Sumi, H., Mihara, H., and Toki, N., Thromb. Haemostas. *48* (1982) 342.
15 Sumi, H., Sasaki, K., and Muramatu, M., Acta haemat., jap. *41* (1978) 766.
16 Claeson, G., Fribeger, P., Knos M., and Erikson, E., Haemostas. *7* (1978) 76.
17 Chase, J. Jr, and Shaw, E., Biochemistry *8* (1969) 2212.
18 Wohl, R.C., Summaria, L., and Robbins, K.C., J. biol. Chem. *254* (1979) 9063.
19 Weber, K., and Osborn, M., J. biol. Chem. *244* (1969) 4406.
20 Vesterberg, O., and Svensson, H., Acta chem. scand. *20* (1966) 820.
21 Gros, C., and Labouesse, B., Eur. J. Biochem. *7* (1969) 463.
22 Sumi, H., Toki, N., Maehara, S., Kosugi, T., Akazawa, K., Matsuo, O., and Mihara, H., Acta haemat. *67* (1982) 263.
23 Sumi, H., Takada, Y., and Takada, A., Thromb. Res. *11* (1977) 747.
24 Sumi, H., Toki, N., Takasugi, S., Maehara, S., Maruyama, M., Akazawa, K., Matsuo, O., and Mihara, H., Thromb. Haemostas. *47* (1982) 14.

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## Presence of serum proteins in submaxillary duct perfusate

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*Summary.* The secretory activity of the main excretory duct of rat submaxillary gland was investigated by the technique of luminal perfusion. Immunologic studies of the perfusate revealed the presence of serum antigens and the absence of intrinsic submaxillary gland antigens. It is suggested that the submaxillary duct permits passive transport of serum proteins to saliva from serum.
*Key words.* Rat submaxillary gland; saliva; serum proteins; submaxillary duct perfusion; serum antigens.

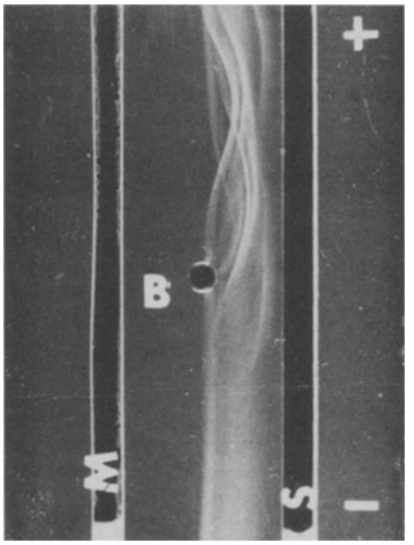
Recent studies have shown evidence of appreciable transport of electrolytes in the main excretory duct of the rat submaxillary gland[1]. Physiological investigations using the technique of luminal perfusion have shown that the rat submaxillary duct actively absorbs sodium and secretes potassium and bicarbonates, and is capable of converting isotonic perfusion fluid to a hypotonic end product[2-4]. In the present study the technique of luminal perfusion was utilized in an effort to evaluate the role of the main submaxillary duct in the secretion of proteins.

*Materials and methods.* Adult male Long-Evans rats, 4–6 months old, were used in the experiments. The animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). For perfusion of the main excretory duct of the submaxillary gland the surgical procedure previously described was followed[5]. The oral end of the right submaxillary duct was first cannulated, using fine polyethylene tubing (Clay-Adams PE 10), to a depth of approximately 3 mm. An opening was made in the skin over the submaxillary-sublingual gland complex, and the hilar end of the submaxillary duct was separated from the sublingual duct. A diagonal cut was made in the submaxillary duct, and a polyethylene cannula with a tip diameter of approximately 100 µl, or less, was inserted in the lumen and ligated in place. The trachea was cannulated to maintain a clear airway.

The submaxillary duct was perfused in situ using a Harvard pump set to deliver fluid from a 0.5-ml glass syringe at a constant rate of 162 nl/min. Normal saline was used for perfusion. Samples of perfusate were collected by attaching short lengths of PE 50 tubing over the PE 10 tubing of the oral cannula and 10-µl micropipettes were used for collecting the samples. Perfusion continued for 60 min for each animal. In some experiments perfusion was carried our after injection of isoproterenol HCl (5–6 mg/rat, i.p.).

Antisera were prepared by injecting saline extract of submaxillary gland s.c. into rabbits as previously described[6]. The extract was incorporated into Freund's adjuvant for the first injection and into incomplete adjuvant for subsequent injections. Antiserum to rat serum was prepared in rabbits in a similar manner. Antiserum to rat submaxillary gland extract was absorbed with lyophilized rat serum in a concentration of 80 mg/ml, which was found by preliminary gel diffusion tests to be a suitable concentration for neutralization. Total protein content of perfusion samples was determined by the method of Lowry et al.[7] using bovine serum albumin for establishing a standard curve. Immunoelectrophoresis was carried out according to the method of Grabar and Burtin[8] as modified by Scheideggar[9] using 1% agarose in 0.05 M barbital buffer at pH 8.6. After the antigens were placed in the wells, a constant current of 4 mA per slide was applied for 2 h. Diffusion of antisera was allowed to proceed for 2–3 days.

*Results.* Immunoelectrophoretic studies of the submaxillary duct perfusate exhibited several lines of precipitation with antisera to extracts of rat submaxillary gland. Absorption of the antisera with lyophilized rat serum abolished the reaction with




Immunoelectrophoresis in agar gel. Antisera in troughs: anti-submaxillary gland extract absorbed with rat serum (M); antirat serum (S). Well B contains sample of submaxillary duct perfusate. Note absence of intrinsic antigens of submaxillary gland origin and the presence of serum antigens.