A Trypsin-Like Protease in Guinea-Pig Skin

We are interested in determining the endogenous chemical mediators responsible for the biphasic increase in vascular permeability in the irradiation-induced inflammatory skin[1], and reported that some of the protein fractions obtained from normal or irradiated guinea-pig skin extract caused an inflammatory response and a relatively prolonged increase in vascular permeability to plasma protein[2]. The increase in vascular permeability in inflammed skin is, at least in part, due to the liberation of bradykinin-kallidin-type vasoactive peptides from their precursor, α_2-globulin, by a trypsin-like protease[3,4]. However, attempts to isolate and identify these trypsin-like enzymes in mammalian skin have been unsuccessful, although the presence of other proteases such as chymotrypsin or SH-dependent protease have been reported by many workers[5-9]. In this communication we describe our finding of the presence of a trypsin-like protease in the extract of guinea-pig skin.

Skin of closely clipped flanks of normal albino guinea-pigs (2 months old, 400–500 g) was cut into small fragments, frozen with dry ice, and smashed with a stainless steel pulverizer. This was defatted with 3 changes of cold acetone, dried under a hood, and kept over P_2O_5 in vacuo overnight. The resultant white powder was extracted with 10 volumes of cold 0.067 M phosphate buffer, pH 7.4, for 4 h with continuous stirring, and the suspension centrifuged for 30 min at 10,000 g. The supernatant was dialyzed against several changes of 100 volumes of buffer and chromatographed on Sephadex G-50[10]. Fractions I and II as shown in Figure 1 were pooled separately, dialyzed against several changes of a large volume of deionized water to remove salts, lyophilized, and stored at −20°C until used. All these procedures were performed at or below 4°C.

Protein concentration was measured by the method of LOWRY et al.[11] using bovine serum albumin as standard, and proteolytic activity was determined with casein as substrate[12]. Fractions I and II (Figure 1) actively hydrolyzed casein at pH 7.4. The specific proteolytic activity of fraction II was about 5–7 times greater than that of fraction I.

Esterolytic activity was studied using a minor modification of the SCHWERT and TAKENAKA's method[13]. The 3 ml of 0.1 M phosphate buffer, pH 7.6, containing enzyme and $5 \times 10^{-4} M$ N-(α)-benzoyl-L-argine ethyl ester (BAEE, Sigma Chemical Co., St. Louis) and a blank containing buffer and substrate alone were incubated at 37°C with agitation for one to several hours. The difference in optical density at 254 nm between the reaction mixture and the blank was recorded with a Hitachi P-E 139 spectrophotometer. Under these conditions, an increase in optical density of 0.345 corresponded to the hydrolysis of 1 μmole of BAEE. The fraction II had no detectable esterase activity with BAEE substrate. However, the rate of BAEE hydrolysis by fraction I was directly proportional to enzyme concentration up to 0.8 mg of enzyme protein in 3 ml of $5 \times 10^{-4} M$ BAEE solution. Its Km value was $2.1 \times 10^{-4} M$. There was a broad pH optimum for hydrolysis of BAEE ranging between 7.2 and 9.0 with a possible maximum at pH 8.5. This activity was slightly lower in *Tris* buffer.

Preincubation of 1.2 mg of fraction I in 1 ml of buffer with 300 μg of soy bean trypsin inhibitor (SBTI, Nutritional Biochemical Corp., Cleveland) ($1.67 \times 10^{-5} M$) or 300 units of Trasylol[14] (FBA Pharmaceutical Inc., New York) at 37°C for 30 min resulted in 50% and 70% inhibition of esterase activity (Figure 2). In the study of inhibitory effect of 1-chloro-3-tosylamido-7-amino-L-2-heptanone (TLCK) (Calbiochem, Los Angeles), enzyme

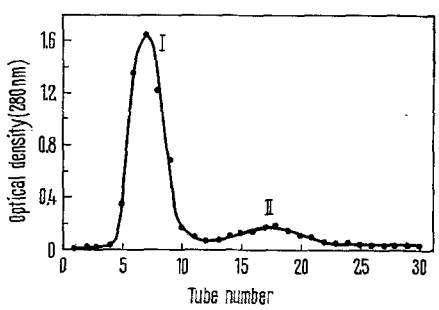

Fig. 1. Chromatography of skin extract on a 2.3 × 35 cm Sephadex G-50 (fine) column. The eluent was 0.067 M phosphate buffer, pH 7.4, and the flow rate was 5 drops per minute; 150 drops per tube.

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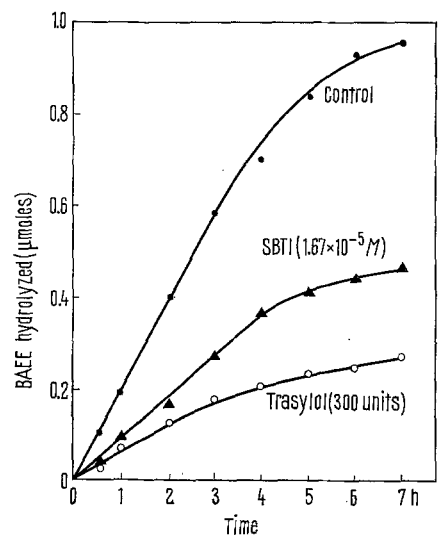

Fig. 2. Inhibitory effect of SBTI and Trasylol. Assay mixture contained 0.6 mg of enzyme protein and $5 \times 10^{-4} M$ BAEE in 3 ml of 0.1 M phosphate buffer, pH 7.6.

plus TLCK or enzyme alone were incubated for 3 h at room temperature and dialyzed against buffer to remove the free TLCK before the enzyme assay. Because of instability of TLCK at high pH, phosphate buffer pH 7.0 was used. TLCK with the concentration of $1.71 \times 10^{-3} M$ and $5.12 \times 10^{-3} M$ per 2.3 mg of protein caused partial or almost complete inhibition (about 90%) of fraction I esterase activity (Figure 3).

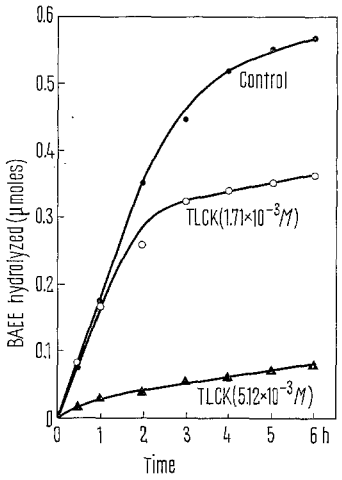

Fig. 3. Inhibitory effect of TLCK, a specific trypsin inhibitor. Fraction 1, 2.3 mg, was preincubated in 3 ml of $1.71 \times 10^{-3} M$ (1.7 mg) or $5.12 \times 10^{-3} M$ (5.1 mg) TLCK. Assay mixture contained 0.6 mg of protein and $5 \times 10^{-4} M$ BAEE in 3 ml of $0.1 M$ phosphate buffer, pH 7.0.

The hydrolysis of BAEE, one of the typical trypsin substrates[15], and the inhibition of this esterase activity by SBTI, Trasylol and TLCK strongly suggest that fraction I contains trypsin or trypsin-like protease(s). The inhibitory effect of TLCK on the esterase activity of fraction I is particularly significant in support of our contention since, contrary to SBTI and Trasylol which inhibit not only trypsin but also other proteases including chymotrypsin[16,17], TLCK inhibits only trypsin (Figure 4); TLCK effectively suppressed trypsin activity, whereas even higher concentration of TLCK had no effect on α-chymotrypsin. SHAW et al.[18] reported that TLCK specifically inactivated trypsin stoichiometrically by irreversibly binding with the histidine residue in the active site, and that it had no effect on chymotrypsin.

The intradermal injection of fraction I in guinea-pig skin results in an inflammatory reaction and a prolonged (3 h) increase in vascular permeability to plasma protein[2]. The mechanism for this reaction remains to be determined, but it is reasonable to assume that a release of bradykinin-kallidin-type peptides from their circulating precursors may occur due to the trypsin-like proteolytic activity of fraction 1. Since this fraction is only partially purified, it is quite possible that it contains other than trypsin-like protease(s). Further purification and characterization of its enzymatic and vasoactive properties are in progress.[19]

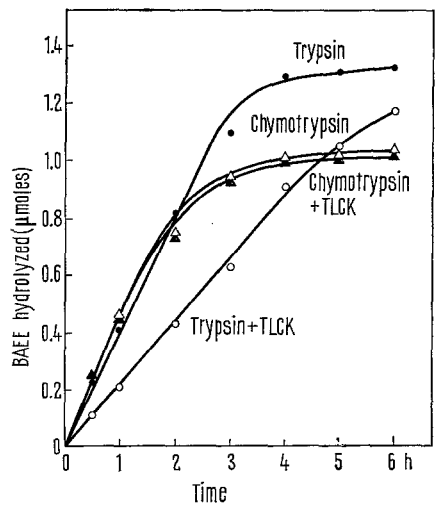

Fig. 4. Inhibitory effect of TLCK on trypsin and α-chymotrypsin. Trypsin, 35 μg ($2.92 \times 10^{-7} M$) was preincubated in 5 ml of $6.03 \times 10^{-6} M$ TLCK and 200 μg ($1.74 \times 10^{-6} M$) of α-chymotrypsin was preincubated in 5 ml of $1.21 \times 10^{-4} M$ TLCK. Other experimental conditions were as in Figure 3 except that the aliquot of the above mixture contained 0.7 μg of trypsin or 100 μg of α-chymotrypsin for the assay.

Zusammenfassung. Eine der vaso-aktiven Proteinfraktionen, aus Meerschweinchenhautextrakt gewonnen, hydrolisiert Kasein und N-(α)-benzoyl-L-Arginin Ethyl Ester. Substratspezifität, optimaler pH-Wert und Inaktivierung durch eine speziellen Trypsininhibitor (TLCK) beweisen, dass diese Proteinfraktion eine trypsinähnliche Protease enthält.

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Hemmeffekt von Harnstoff auf die durch Insulin stimulierte Glykogenbildung in zwei verschiedenen Muskelpräparaten in vitro[1]

Die stimulierende Wirkung von Insulin auf die C-1-Oxydation von Glukose-1-C^{14} im epididymalen Fettgewebe wird durch Harnstoff kompetitiv gehemmt[2]. In den folgenden Experimenten wurde untersucht, ob Harnstoff auf den durch Insulin stimulierten Glukosestoffwechsel des Muskelgewebes eine vergleichbare Hemmung ausübt. Als Mass der Insulinwirkung diente die Glykogenbildung. Für die Untersuchungen wurde das Diaphragma