CHROM. 5289

Removal of chymotrypsin activity from crystalline trypsin and its investigation

During the course of our studies on the primary structure of melittin[1,2] we were faced with the problem of locating one tryptic peptide in the melittin sequence. Peptide T-1/1 is formed in good yield by unspecific cleavage at the Try–Ile bond of melittin by commercial trypsin preparations. This is due to chymotrypsin-like contamination, which has been observed in several other hydrolytic studies on polypeptide substrates[3–9].

A critical survey of the work in the last decade on the chromatographic removal of inert proteins or other enzymes in commercial trypsin preparations[10–17] reveals that the tryptic activity of such preparations may be increased. But no investigation on the chymotrypsin-like contamination of these trypsin preparations has been reported in the publications cited above.

Because of its specific inhibition of chymotrypsin, L-(α-tosylamido-β-phenyl)-ethyl-chloromethylketone (TPCK) has also been studied in purification experiments[5,18]. After incubation of trypsin with TPCK the remaining inhibitor was removed by dialysis. It is clear that an appreciable chymotryptic activity against acetyl-L-tyrosine ethyl ester still remains in the trypsin if incubation and dialysis are the only purification procedures. MAROUX *et al.*[18] assumed that pure trypsin is able to split certain aromatic bonds in high molecular weight peptides at an appreciable rate.

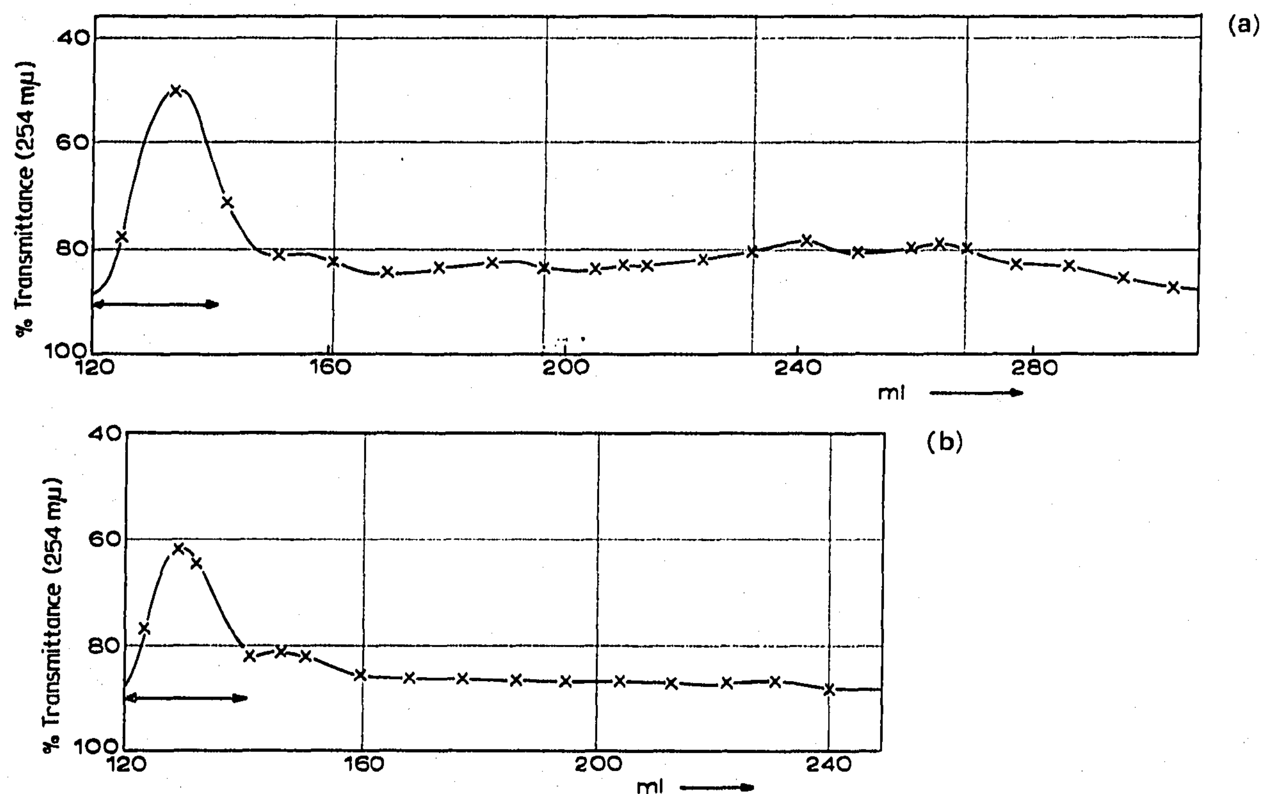

Fig. 1. (a) Chromatography of TPCK-treated bovine trypsin on Sephadex G-50. (b) Rechromatography of the main peak (pooled tubes denoted by the double-headed arrow).

TABLE I

TRYPTIC DEGRADATION PRODUCTS OF MELITTIN

Peptides are designated by the prefix T to indicate their origin from a tryptic digest. The first fraction number indicates the sequence of elution from the ion exchange column[2] and the second stands for elution from paper, beginning near the front. Chymotrypsin-free trypsin is highly specific for arginine and lysine residues and cleaves only these peptide bonds. Chymotrypsin contaminated trypsin results in similar peptide patterns and, in addition, varying amounts of contaminating peptides, such as T-1/1, T-3/1, and T-4/1. Peptide T-1/1 substitutes T-X from the former peptide maps.

	(X)- H-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Try-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH_2
T-2/2	H-Gly-Ile-Gly-Ala-Val-Leu-Lys
T-1/2	X-Gly-Ile-Gly-Ala-Val-Leu-Lys
T-X	Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Try-Ile-Lys
T-1/1	Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Try
T-3/1	Ile-Lys
T-4/1	Ile-Lys-Arg
T-3/2	Arg
T-5	Lys-Arg
T-2/3	Gln-Gln-NH_2

In this publication I wish to report a simple chromatographic procedure for removal of chymotrypsin-like impurities from TPCK-treated trypsin.

TPCK-treatment

An anhydrous methanolic solution (0.5 ml) of TPCK (25 mg) (Calbiochem, Luzern) was added to 90 mg crystalline bovine trypsin (No. 37260, Serva, Heidelberg G.F.R.) dissolved in 27 ml 0.01 *M* $CaCl_2$. The reaction mixture was incubated for 5 h at room temperature and occasionally stirred. The pH was maintained at 7.0 by addition of 0.5 *N* NaOH, which was monitored with the aid of a Metrohm pH-stat. After removal of the precipitate the clear solution was immediately lyophilized.

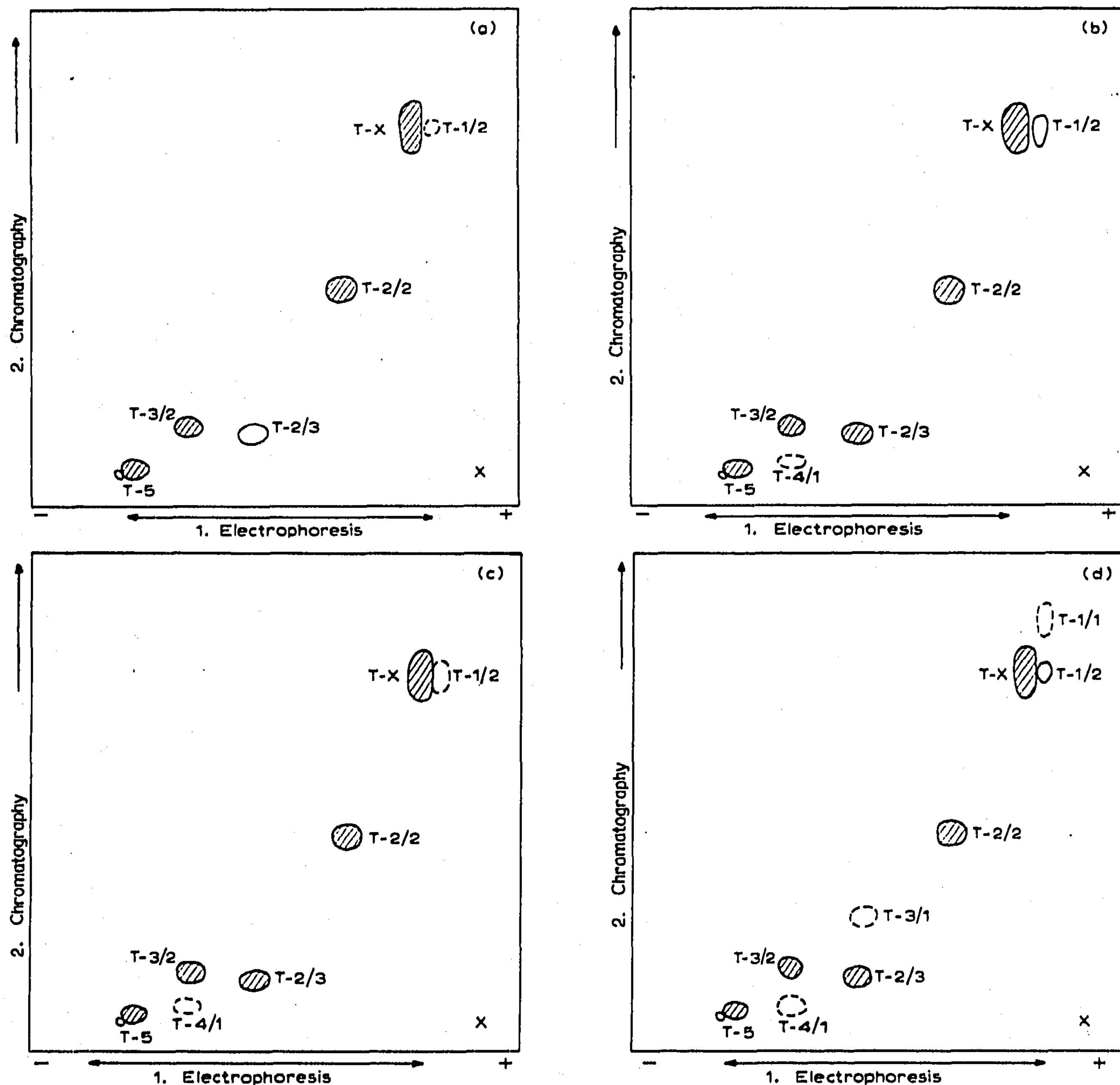

Fig. 2.

(*Continued on p. 453*)

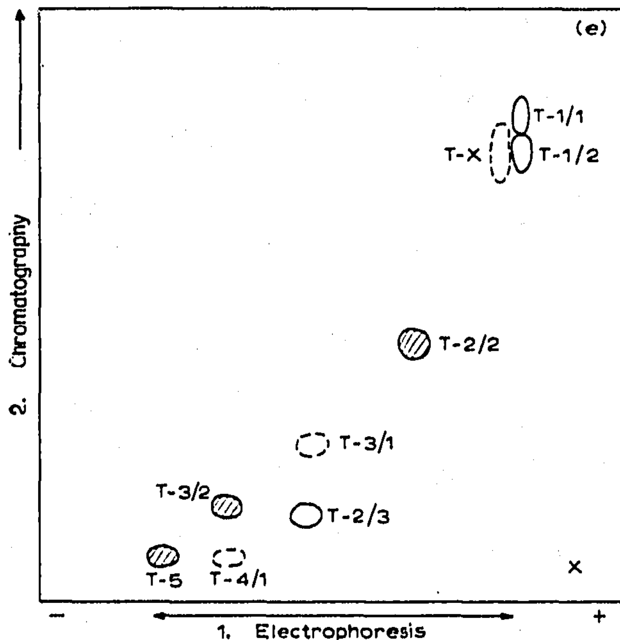

Fig. 2. Tracing of tryptic fingerprints of melittin with the following trypsin preparations: Bovine trypsin TPCK-treated and twice chromatographed on Sephadex G-50 (a), trypsin "TRPTCK" from Worthington (b), trypsin "TRTPCK", 210 U/mg, from Seravac (c), bovine trypsin "DCC)-treated", Type XI, from Sigma (d), and native untreated porcine trypsin from Merck, Darmstadt (e). All five fingerprints were run simultaneously under identical conditions. The peptides were stained with ninhydrin and the relative intensities of staining are indicated by the heaviness of shaded spots. See text for other details.

Gel filtration

A column (1.9 × 100 cm) of Sephadex G-50 fine (Pharmacia, Uppsala, Sweden) was equilibrated with 0.05 *M* ammonium formate (pH 8.0) (0.05 *M* formic acid was adjusted to pH 8.0 with ammonia and deaerated under aspiratory vacuum for 2–4 min). The lyophilized trypsin preparation was dissolved in 3 ml water and one drop 0.5 *N* NaOH and applied to the column. After washing the residual trypsin with 1 ml buffer and applying it to the column, the enzyme was eluted with the same buffer. The fractions were collected at a flow rate of 18 ml/h at 4°, pooled and lyophilized. The effluent was monitored for protein by measuring the transmittance at 254 mμ. The preparation was rechromatographed on Sephadex G-50 fine in the same manner as before. Yield: 18 mg of the purified trypsin (Fig. 1). The low molecular material may represent proteins and peptides resulting from autolysis of the trypsin in the course of incubation, lyophilization, and chromatography[19].

Tryptic digestion of melittin.

1 mg melittin was dissolved in 0.2 ml 0.1 *M* NH_4HCO_3 buffer (pH 7.9) (0.1 *M* ammonium carbaminate was adjusted to pH 7.9 with HCOOH) and mixed with 0.2 ml of a trypsin solution to give an enzyme to substrate ratio of 1:50 by weight. (Preparation of the trypsin solution: 0.1 mg trypsin in 20 μl 0.05 *N* HCl was mixed with 0.98 ml 0.1 *M* NH_4HCO_3 buffer, pH 7.9). The mixture was incubated for 6 h at 37°, after which the reaction was terminated by adding 0.1 ml 1 *M* HCOOH, and the solution was lyophilized. The product was redissolved in 0.5 ml water and lyophilized. The pure white powder was dissolved in 100 μl water.

Fingerprint

15–20 µl of the aqueous hydrolysate were used on a sheet of Whatman No. 1 paper. Electrophoresis was carried out at pH 5.8 in pyridine–acetic acid–water (40:6:1000, v/v) for 1 h 10 min at 50 V/cm. Descending chromatography was run in *n*-butanol–acetic acid–water (4:1:5, v/v) for 12 h. The peptides were stained with 0.3 % ninhydrin (w/v) in water-saturated *n*-butanol containing 5 % collidine and 10% acetic acid, and developed at 110° for 5 min (Fig. 2).

As shown in Fig. 2 and Table I the purest enzyme preparation was the bovine trypsin which had been TPCK-treated and twice chromatographed as described above. All the other trypsin preparations investigated showed varying amounts of atypical peptides as a result of chymotryptic digestion. The peptide map in Fig. 2e shows appreciable amounts of peptides T-1/1, T-3/1, and T-4/1, indicating, that native untreated porcine trypsin[19], prepared by the method of TRAVIS AND LIENER[20], is not useful for structural studies on polypeptides and proteins. This is in contrast to results published by TRAVIS[19], claiming untreated recrystallized porcine trypsin to be essentially free of chymotrypsin activity and suggesting its suitable application instead of TPCK-treated bovine trypsin for exclusively tryptic digestion of polypeptides and proteins.

The author is indebted to Serva (Heidelberg) and Merck (Darmstadt) for the kind gift of samples of trypsin.

Department of Biochemical Pharmacology, J. JENTSCH
Max-Planck Institute for Experimental Medicine,
Herman Rein.Str. 3, 34 Göttingen (G.F.R.)

1 J. JENTSCH AND E. HABERMANN, in H. C. BEYERMAN *et al.* (Editors), *Peptides*, North-Holland, Amsterdam, 1967, p. 263.
2 J. JENTSCH, *Z. Naturforsch.*, 24b (1969) 264.
3 W. W. BROMER, A. STAUB, L. G. SINN AND O. K. BEHRENS, *J. Amer. Chem. Soc.*, 79 (1957) 2801.
4 T. INAGAMI AND J. M. STURTEVANT, *J. Biol. Chem.*, 235 (1960) 1019.
5 V. KOSTKA AND F. H. CARPENTER, *J. Biol. Chem.*, 239 (1964) 1799.
6 K. TAKAHASHI, *J. Biol. Chem.*, 240 (1965) PC 4117.
7 S. MAROUX AND M. ROVERY, *Biochim. Biophys. Acta*, 113 (1966) 126.
8 S. MAROUX, A. PUIGSERVER, V. DLOUHA, P. DESNUELLE, G. H. DE HAAS, A. J. SLOTBOOM, P. P. M. BONSEN, W. NIEUWENHUIZEN AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 188 (1969) 351.
9 H. TSCHESCHE AND E. WACHTER, *Eur. J. Biochem.*, 16 (1970) 187.
10 H. F. BUNDY AND J. MEHL, *J. Clin. Invest.*, 37 (1958) 947.
11 I. E. LIENER, *Arch. Biochem. Biophys.*, 88 (1960) 216.
12 R. D. COLE AND J. M. KINKADE, *J. Biol. Chem.*, 236 (1961) 2443.
13 S. MAROUX, M. ROVERY AND P. DESNUELLE, *Biochim. Biophys. Acta*, 56 (1962) 202.
14 M. CHARLES, M. ROVERY, A. GUIDONI AND P. DESNUELLE, *Biochim. Biophys. Acta*, 69 (1963) 115.
15 P. O. GANROT, *Acta Chem. Scand.*, 20 (1966) 175.
16 S. PAPAIOANNOU AND I. E. LIENER, *J. Chromatogr.*, 32 (1968) 746.
17 M. TUNIS, *Science*, 162 (1968) 912.
18 S. MAROUX, M. ROVERY AND P. DESNUELLE, *Biochim. Biophys. Acta*, 122 (1966) 147.
19 J. TRAVIS, *Biochem. Biophys. Res. Commun.*, 29 (1967) 294.
20 J. TRAVIS AND I. E. LIENER, *J. Biol. Chem.*, 240 (1965) 1967.

Received January 22nd, 1971.