IDENTIFICATION OF THE HISTIDINE RESIDUE AT THE ACTIVE CENTER OF TRYPSIN LABELLED BY TLCK*

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Proof for the essential role of histidine in the function of trypsin was provided by the active-site directed agent TLCK, the chloromethyl ketone derived from a trypsin substrate, tosyl-L-lysine (Mares-Guia and Shaw, 1963; Shaw, Mares-Guia, and Cohen, 1965). TLCK inactivates trypsin by stoichiometric alkylation at N-3 of a histidine residue (Petra, Cohen, and Shaw, 1965). As in the earlier work with chymotrypsin (Ong, Shaw, and Schoellmann, 1965) it was desirable to isolate a peptide containing the alkylated histidine residue in order to establish its location in the primary sequence. Such a procedure is described which establishes His-46 as the site of alkylation. There was added interest in this problem in view of the remarkable homology in the covalent structures of the two proteolytic enzymes (Walsh and Neurath, 1964a).

It had been observed (Petra et al., 1965) that in TLCK-inhibited trypsin there is an instability of the inhibitor moiety leading to the liberation of toluenesulfonamide that complicates characterization of the product, but this could be minimized by a borohydride reduction of the ketone group. The procedure described below for the isolation of a TLCK-containing active center peptide takes advantage of this observation.

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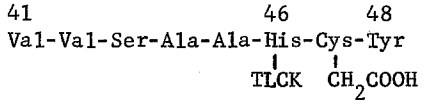
Examination of the primary sequence of bovine trypsinogen (Walsh et al., 1964b; Mikes et al., 1966) suggested that purification of the N-terminal tryptic peptide of alkylated trypsin which contains the two histidine residues analogous to those in chymotrypsin would be a good starting point because of its exceptional size (43 residues). Using TLCK-C^{14} it was established that in a tryptic digest of reduced, carboxymethylated TLCK-C^{14}-inhibited trypsin the radioactivity was largely (85%) precipitated by 10% trichloroacetic acid and that in subsequent gel filtration purification of the precipitate, the radioactivity migrated with the largest fragments. Chymotryptic digestion was then used for further degradation with fractionation of the digest on Dowex-1 with a descending pH gradient (Schroeder et al., 1962). A single radioactive peak was eluted near pH 6 which, when examined by paper electrophoresis and autoradiography was shown to contain a single labelled peptide along with a nonradioactive one. The radiopeptide accounted for 26% of the radioactivity in the reduced, carboxymethylated enzyme used as starting material. Quantitative amino acid analysis of labelled peptide eluted from a paper electrophoresis strip (Table 1) corresponded to the octapeptide



comprising residues 41 to 48 and containing histidine-46 (trypsinogen numbering, Walsh et al., 1964b). Histidine-46 is thus an essential catalytic residue of the active center of trypsin and is exactly analogous in its position within an intrachain disulfide loop to the histidine residue in chymotrypsin which is alkylated by TPCK, the chloromethyl ketone derived from tosyl-L-phenylalanine as cited above (Ong et al., 1965; Smillie and Hartley, 1966). From the structural homology of trypsin and chymotrypsin (Walsh and Neurath, 1964a) as well as the mechanistic similarities (Bender et al., 1964), this was the expected outcome.

Tomasek, Severin, and Sorm (1965) noted earlier that it was

possible to recover only two of the three histidines from a peptic digest of S-sulfo TLCK-inhibited trypsin. The absence of the histidine-46 containing peptide was offered as evidence for the site of attachment.** The present work provides positive evidence for the location of the active center histidine through characterization of a peptide containing isotopically labelled inhibitor. The methods used should be of value in studying the active center of other trypsins† and enzymes of trypsin-like specificity which abound in nature and which can be expected to undergo modification by TLCK.

TLCK-C^{14} prepared from uniformly labelled L-lysine-C^{14} was used to inhibit trypsin as described earlier (Shaw et al., 1965). The inactivated enzyme was purified by chromatography (Schroeder and Shaw, to be published) which was considered desirable for determining the specific activity of the alkylated enzyme but this is not essential for the following isolation procedure which has also been carried out with nonchromatographed material.

For reduction of the ketone group, TLCK-C^{14} inhibited trypsin (441 mg) in water (12 ml) was treated with sodium borohydride (30 mg) at room temperature for 30 minutes, brought to pH 4 with acetic acid, and diluted to 44 ml with water. Solid urea was added to achieve a 10 M solution and reduction and carboxymethylation was carried out as described by Canfield and Anfinsen (1963). The product was isolated by gel filtration on a Biogel P-2 column (60 x 4.3 cm, 50-100 mesh) with elution by

**Conclusions about the site of chemical modification based on recovery of unmodified regions of protein are particularly unreliable in the case of trypsin since a large proportion of typical trypsin preparations (up to 50%, Bender et al., 1965) consist of inert protein. This material will not combine with TLCK (Shaw et al., 1965) yet, being derived from pure trypsinogen can be expected to provide peptides from all regions of trypsin on degradation which, of course, are not coming from the modified enzyme. The histidine content of the TLCK-inhibited trypsin of Tomasek et al. (1965) suggests a content of unmodified trypsin of about 33%. The yields of recovered peptides were not given.

†Preliminary results on TLCK-inhibited porcine trypsin (Smith and Liener, 1967) indicate a similar result to that from the bovine enzyme.

0.001 N HCl. The specific activity was determined to be 1.7×10^4 DPM per micromole of trypsin using amino acid analysis to establish the molarity of trypsin in conjunction with the known composition (Walsh and Neurath, 1964a).

The reduced carboxymethylated alkylated enzyme as a 1% (w/v) suspension in water was digested with 2% (w/w) of TPCK-trypsin (Kosta and Carpenter, 1964) at pH 8, 23°, for 3 hours with stirring and addition of 0.1 N NaOH as needed; digestion was continued for 18 hours at 37° with an additional 1% of TPCK-trypsin and a layer of toluene. An equal volume of 20% trichloroacetic acid was added to the peptide mixture and, after one hour at room temperature, the precipitate was collected at 2000 RPM, washed with 10% trichloroacetic acid and water. The insoluble peptides were suspended in a small volume of water and extracted 3 times with 3 volumes of ether.

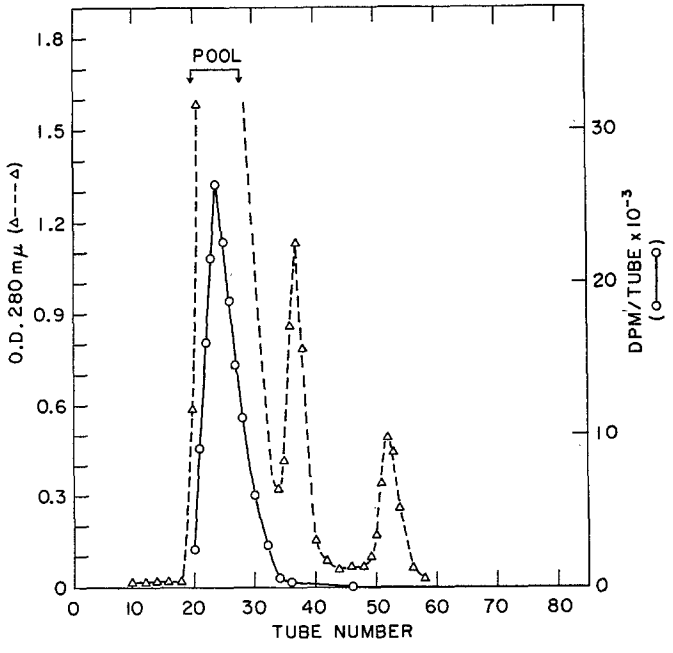


Fig. 1. Gel filtration of TCA-insoluble tryptic peptides from reduced carboxymethylated TLCK-C^{14}-inhibited trypsin on Biogel P-4 (1.8 x 193 cm); elution with 20% formic acid.

The insoluble tryptic peptides were dissolved in 90% formic acid (5 ml), diluted to about 30% formic acid and applied to a Biogel P-4 column (1.8 x 193 cm, 100-200 mesh) for elution with 20% formic acid. The residue from the radioactive peak (Fig. 1) was redissolved in 20% formic acid (10 ml), applied to Biogel P-10 (1.8 x 136 cm, 50-150 mesh) and eluted with the same solvent. The peak tubes were again pooled, evaporated to dryness, redissolved in water, and adjusted to pH 8 for digestion with an estimated 2% (w/w) of α-chymotrypsin at room temperature for about 24 hours with stirring. The pH was maintained with 0.1 N NaOH and a small amount of toluene was added.

An aliquot of the chymotryptic digest (about 3 μmoles in 6 ml) was adjusted to pH 9.8 and centrifuged. A very small precipitate was

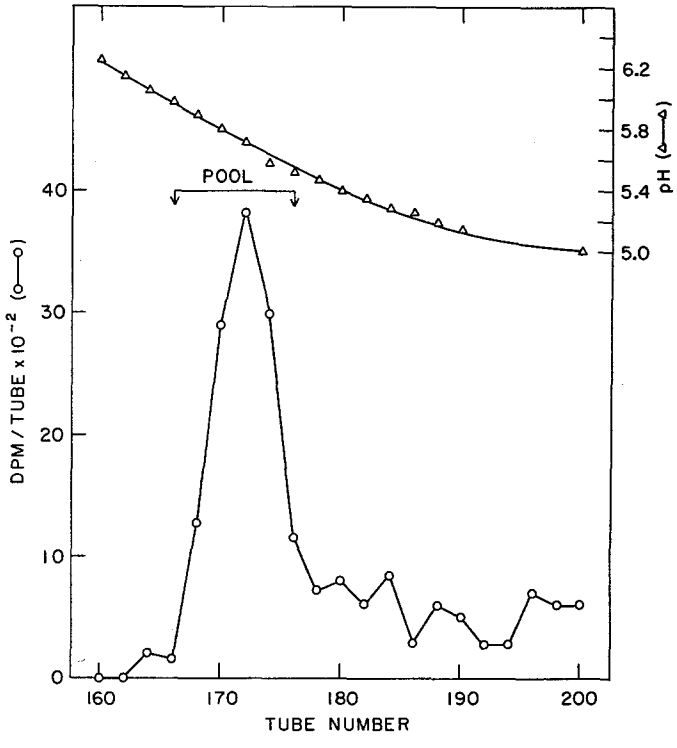


Fig. 2. Ion exchange chromatography of radioactive octapeptide form TLCK-C^{14}-trypsin on Dowex-1 (1.2 x 55 cm) in a descending pH gradient. (Only the portion of the elution curve containing radioactive material is shown.)

discarded and the clear supernatant was applied to a Dowex-1, X-2 column (1.2 x 55 cm) which had been equilibrated with a pH 8.47 volatile buffer (Schroeder and Robberson, 1965). Elution was accomplished with a gradient of volatile buffers between pH 8.47 and pH 4.25 in two stages, using two open vessels of equal volumes, connected at the bottom. Stage 1 consisted of 400 ml pH 8.47 buffer in the mixer and 400 ml pH 6.48 buffer in the reservoir. When the 800 ml had been used up, stage 2 followed with 500 ml pH 6.48 buffer in the mixer and 500 ml pH 4.25 buffer in the reservoir. The peak emerged between about pH 6.0-pH 5.5 (Fig. 2) and was evaporated to a small volume. Following paper electrophoresis at 2000 volts for 90 minutes at pH 3.5 (positive → negative), radioautography showed one radioactive band which was cut out, eluted with 50% ethanol-water, and hydrolyzed with acid. Quantitative amino acid analysis (Spackman, Stein, and Moore, 1958) and radioactivity determination gave the result indicated in Table 1.

TABLE 1

Composition of Isolated Radioactive Peptide

Amino Acid	Observed Molar Ratio	Theoretical Molar Ratio Residues 41-48
Histidine‡	(1)	1
Carboxymethylcysteine	0.72	1
Serine	1.01	1
Alanine	2.21	2
Valine	1.37	2
Tyrosine	0.69	1
All others	< 0.07	0

‡Based on specific activity of labelled protein. Analyses carried out by H. G. Latham.

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