# INHIBITION OF FICIN BY THE CHLOROMETHYL KETONE DERIVATIVES OF N-TOSYL-L-LYSINE AND N-TOSYL-L-PHENYLALANINE*

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The most definitive evidence for the participation of certain amino acid residues in the active site of enzymes has come from the use of specific inhibitors. Thus, DFP*** is known to inhibit a number of animal proteases by reacting with a unique serine residue (Hartley, 1960), and the chloromethyl ketone derivatives of N-tosyl-L-phenylalanine (TPCM) and N-tosyl-L-lysine (TLCM) stoichiometrically inactivate chymotrypsin and trypsin respectively by alkylation of a single histidine residue (Schoellmann and Shaw, 1963; Shaw et al., 1965). The remarkable similarity of the amino acid sequences surrounding these two amino acids in several of the animal proteases suggests a common catalytic site for the hydrolytic activity of these enzymes (Walsh et al., 1964; Smillie and Hartley, 1966).

Plant proteases such as papain, ficin, and bromelain have been generally regarded as SH-enzymes in which cysteine replaces serine as the nucleophilic site of acylation by the substrate (Smith, 1958; Hammond and Gutfreund, 1959;

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*** Abbreviations: DFP, diisopropylphosphofluoridate; TPCM: N-α-tosyl-L-phenylalanyl-chloromethane; TLCM: N-α-tosyl-L-lysyl-chloromethane; BAL: 2,3-dimercapto-1-propanol.

Lowe and Williams, 1965a). The involvement of histidine at the active site of these so-called thiol proteases has been postulated mainly on the basis of kinetic evidence (Lowe and Williams 1965b, 1965c), although Shaw et al. (1965) make a brief footnote reference to the fact that papain could be inhibited by both TPCM and TLCM. We have made a similar observation with ficin and wish to report further that, contrary to expectations, the thiol group of this enzyme is the site of reaction with these inhibitors.

## Experimental

Ficin was prepared and activity measurements were made using casein as the substrate (Liener, 1961). The molar concentration of the enzyme solutions was based on nitrogen content, determined by the method of Lanni et al. (1950), multiplied by 6.25, and a molecular weight of 26,000 (Bernhard and Gutfreund, 1956). Inhibition experiments were conducted by adding 1 ml of 0.001 M EDTA, pH 4.6, containing $1.29 \times 10^{-3}$ μmole ficin to 1 ml of 0.1 M Na phosphate buffer, pH 6.5, into which various levels of TLCM (Cyclo Chemical Corp.) or TPCM (Calbiochem) had been incorporated. The solutions were allowed to sit at 4° for 18 hr, and 1 ml aliquots were then removed for measurement of activity.

Amino acid analyses (Spackman et al., 1958) were performed on solutions of ficin which had been treated with a 10-fold molar excess of TLCM or TPCM as described above. Cysteine was determined as carboxymethyl-cysteine following treatment with iodoacetic acid (Brigham et al., 1960). Non-inhibited ficin was run under the same experimental conditions in order to assess the extent to which spontaneous loss of cysteine may have occurred.

## Results

Fig. 1 shows the inhibitory effect of TLCM and TPCM on the proteolytic activity of ficin. Extrapolation of the linear portions of these curves to zero

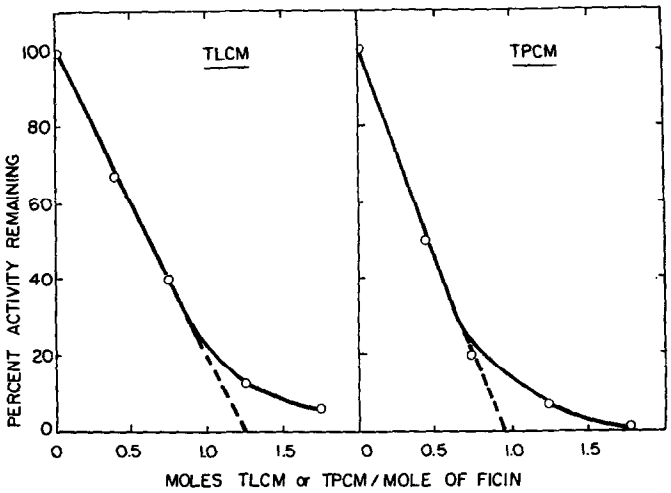


Figure 1. Inhibition of ficin by TLCM and TPCM. Activity in absence of inhibitor taken as 100%.

activity indicates that the reaction of these reagents with ficin is essentially stoichiometric, i.e. 1 mole of ficin was inhibited by 1.25 mole of TLCM and 0.95 mole of TPCM.

A comparison of the amino acid analyses of the inhibited enzyme with the uninhibited control (Table I) reveals that the only amino acid to have undergone a significant change was cysteine. Chromatographic analysis failed to reveal the derivative resulting from the alkylation of cysteine by TPCM and TLCM. This may be due to the high affinity of the p-toluenesulfonamido group for sulfonated polystyrene resin employed for the amino acid analysis (Petra et al., 1965). It is nevertheless evident that the reaction of ficin with TLCM or TPCM had resulted in the loss of the one known reactive SH group in ficin (Liener, 1961). Especially noteworthy is the fact that the 2 histidine residues of ficin were unaffected by treatment with a 10-fold molar excess of either TLCM or TPCM.

Table I.

Amino acid composition* of ficin compared with TLCM- and TPCM-inhibited ficin.

| Amino acid | Ficin | Ficin inhibited with | |
|---|---|---|---|
| | | TLCM | TPCM |
| Lysine | 9.1 | 8.9 | 9.3 |
| Histidine | 2.0 | 2.3 | 2.2 |
| Arginine | 9.8 | 9.7 | 10.1 |
| Aspartic acid | 20.9 | 20.5 | 20.7 |
| Threonine | 9.7 | 9.7 | 9.5 |
| Serine | 16.0 | 16.2 | 15.9 |
| Glutamic acid | 25.1 | 24.9 | 24.6 |
| Proline | 12.9 | 13.3 | 13.1 |
| Glycine | 32.8 | 32.3 | 33.1 |
| Alanine | 20.9 | 20.7 | 20.9 |
| Half-cystine | 7.8 | 7.6 | 7.5 |
| Valine | 19.1 | 19.3 | 18.8 |
| Methionine | 3.8 | 3.6 | 3.7 |
| Isoleucine | 10.2 | 10.4 | 10.1 |
| Leucine | 17.0 | 17.1 | 16.7 |
| Tyrosine | 14.7 | 14.5 | 14.5 |
| Phenylalanine | 6.0 | 5.8 | 5.9 |
| Cysteine** | 0.6 - 0.8*** | 0.04 | 0 |

* Residues per mole, assuming a molecular weight of 26,000 (Bernhard and Gutfreund, 1956).

** Determined as carboxymethyl-cysteine.

*** Values of control ficin solutions were actually 0.64 and 0.79 in the case of the experiments with TLCM and TPCM respectively.

$HgCl_2$ inactivates ficin by reacting with its essential thiol group, and activity can be restored by subsequent treatment with BAL (Liener, 1961). The inactivation of ficin by TLCM or TPCM, however, cannot be reversed with BAL (see Table II). If TLCM and TPCM do in fact react with the thiol group of ficin, then the exposure of ficin to $HgCl_2$ prior to treatment with these reagents should in no way interfere with the ability of BAL to restore its activity. The results of the experiments summarized in Table II fully bear out these expectations.

Table II

The Effect of TLCM and TPCM on the Activity of Ficin Subjected to Prior Treatment with $HgCl_2$. Activity of ficin activated with BAL taken as 100%.

| System | Relative Activity |
|---|---|
| Ficin + BAL | 100 |
| Ficin + $HgCl_2$ | 5 |
| Ficin + $HgCl_2$ + BAL | 100 |
| Ficin + TLCM/TPCM | 0 |
| Ficin + TLCM/TPCM + BAL | 3 |
| Ficin + $HgCl_2$ + TLCM/TPCM + BAL | 98 |

Solutions containing $5.2 \times 10^{-3}$ μmole ficin and $7.4 \times 10^{-3}$ μmole $HgCl_2$ in 0.1 M phosphate buffer, pH 6.5, final volume 3 ml, were allowed to stand at room temperature for 2 hr. One ml of buffer containing $26.4 \times 10^{-3}$ μmole TLCM or $30 \times 10^{-3}$ μmole TPCM was added and the mixture left at 4° for 16 hr. One ml aliquot was then assayed for activity. Appropriate levels of buffer replaced solutions of $HgCl_2$, TLCM, or TPCM in those systems from which these components were omitted. Where activation was desired 0.1 ml of 0.3 M BAL was added to the substrate (casein).

## Discussion

The data presented here support the conclusion that the inactivation of ficin by TLCM and TPCM leads to the loss of its essential thiol group rather than a histidine residue as noted by others for trypsin and chymotrypsin. These findings serve to emphasize the caution expressed by Shaw et al. (1965) that it should not be assumed that these reagents provide a general test for the essentiality of histidine since alkylation of some other important functional group may be favored.

Husain and Lowe (1965) have reported that papain was inhibited by the

chloromethyl ketone of N-tosyl-glycine by virtue of its reaction with the active center cysteine of this enzyme. These authors were of the opinion that this inhibitor was directed to the active site of papain because of its structural similarity to the methyl ester of N-tosyl-glycine which it readily hydrolyzes. The inhibition of papain and ficin by both TLCM and TPCM as well may be a reflection of the broad specificity which these enzymes exhibit (Hartley, 1960). Alternatively, the inhibition of these enzymes by a diverse series of chloromethyl ketones may simply be a consequence of the ease with which thiol groups can be alkylated by α-haloketones (Bartlett, 1953) and may be wholly unrelated to the structural features of the remainder of the molecule.

Cogent arguments in favor of a catalytic mechanism involving the concerted action of a histidine as well as a cysteine residue at the active site of papain have been presented by Lowe and Williams (1965b). The results of the experiments reported here are not necessarily in conflict with this hypothesis. It is known that the loss of a histidine residue of chymotrypsin will not occur with TPCM if its reactive serine residue is first phosphorylated with DFP (Schoellmann and Shaw, 1963). By analogy, the favored alkylation of the cysteine residue of ficin by TLCM or TPCM (or the formation of a mercaptide with $HgCl_2$) could interfere with the potential reactivity of a histidine residue with these same reagents. It would appear that definitive chemical evidence for the catalytic role of histidine in SH-proteases must await the discovery of a reagent which will selectively modify this particular amino acid under conditions wherein the thiol group remains intact.

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