# REACTIVITY OF TRYPTOPHYL RESIDUES IN ELASTASE WITH 2-HYDROXY-5-NITROBENZYL BROMIDE

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## 1. Introduction

In several proteolytic enzymes the substitution of one tryptophyl residue with 2-hydroxy-5-nitrobenzyl bromide (Koshland's reagent) is followed by the complete loss of their proteolytic activity. This is the case of pepsin [1] and of papain [7]. Chymotrypsin is inactivated by the substitution of the tryptophyl residue No. 215 [5]. The same effect occurs when tryptophan No. 199 is substituted in trypsin [2]. Chymotrypsinogen and trypsinogen, substituted in the analogous way can no longer be activated in the corresponding enzymes [3,4]. This selective reactivity of one tryptophyl residue can hardly be explained solely by its exposure on the surface of the enzyme molecule: other tryptophyl residues which when exposed also do not react. On the other hand, the reactive tryptophans in chymotrypsin, trypsin and papain are close to the active sites of these enzymes; a hypothesis has been advanced that the indole ring of the reactive tryptophan is activated by interaction with the imidazole ring of the histidine of the active site [5].

Elastase has an analogous structure with chymotrypsin and trypsin. It contains seven tryptophyl residues, of which No. 12, 39, 132, 164 and 232 are in analogous sites with those in chymotrypsin. In the case of elastase in position 208 there is a phenylalanine residue replacing the tryptophyl residue (No. 215) which was shown to react specifically with Koshland's reagent in chymotrypsin.

We studied recently the reaction of elastase with 2-hydroxy-5-nitrobenzyl bromide in order to compare the reactivity of the tryptophyl residues in elastase with that in chymotrypsin and trypsin. At acidic pH values the conformation of elastase changes profoundly [9]; above pH 5, elastase retains full activity, whereas below pH 4 the enzyme is rapidly inactivated. We therefore studied the reaction at three different pH values in the acidic region.

## 2. Materials and methods

### 2.1. *Enzymes and reagents*

Elastase (EC 3.4.4.7) was a commercial sample (Worthington E.S.F.F.). 2-Hydroxy-5-nitrobenzylbromide was purchased from Fluka, α-*N*-benzoyl-L-alanine-methylester from Sigma.

### 2.2. *Substitution of elastase with 2-hydroxy-5-nitrobenzylbromide*

In a typical assay, 2-hydroxy-5-nitrobenzylbromide (25 mg in 0.1 ml of dry acetone) was added rapidly with vigorous stirring to a solution of 2 mg of elastase in 2 ml of NaCl (0.1 M) $CaCl_2$ (0.02 M) at pH 5.4 in the recipient of a Metrohm autotitrator maintained at 25°C. The pH of the solution was kept at 5.4 ± 0.2 by the addition of 0.05 N NaOH and aliquots were taken for activity assay. After the reaction, the solution was acidified with trichloroacetic acid 5%, the precipitate was collected by centrifugation and washed with acetone to remove the excess of Koshland's reagent.

### 2.3. *Activity measurements*

α-*N*-benzoyl-L-alanine methylester was used as substrate according to Shotton [8]. The pH of the solution (0.1 M Tris HCl–0.01 M in KCl, pH 8.0) was maintained with 0.05 N NaOH in a recording Metrohm autotitrator. A unit of activity is equivalent to a micromole of hydrolyzed substrate per minute at 25°C.

2.4. *Amino acid analysis*

The protein was hydrolyzed with 4-*N*-methanesulfonic acid at 105°C for 20 hr according to Moore [6]. Tryptophan was determined quantitatively using a short column of a Beckman Multichrome apparatus.

## 3. Results and discussion

Elastase is less stable than chymotrypsin or trypsin in an acidic pH range. Therefore the substitution of the native enzyme has been effected at pH 5.4. At this pH the reagent quickly decomposes spontaneously and consequently a large excess (thousand fold) must be used.

As can be seen in table 1, none of the seven tryptophyl residues were modified at this pH. The enzyme remains fully active. Simultaneously with the unfolding of the molecule at more acidic pH values, the activity drops to negligible values and three tryptophanyl residues become accessible to the reagent at pH 4.2. At pH 3.0 the process of unfolding is still not completed because three out of seven tryptophyl residues remain resistant to the substitution.

Table 1
Tryptophan content and activity of elastase after reaction with 2-hydroxy-5-nitrobenzyl bromide at different pH values

| | Untreated Enzyme | Reaction at pH | | | Ref. 8 |
|---|---|---|---|---|---|
| | | 5.4 | 4.2 | 3.0 | |
| Trp | 7.1 | 6.7 | 3.8 | 3.0 | 7 |
| Lys | 3.0 | 3.2 | 3.0 | 3.0 | 3 |
| Arg | 11.4 | 11.6 | 12.2 | 11.6 | 12 |
| Activity U/mg | 12.7 | 13.7 | $7.10^{-3}$ | $7.10^{-3}$ | 13.4 |

The difference between the reactivity of tryptophyl residues in elastase and chymotrypsin is in good agreement with the difference in their structure. The only reactive tryptophan in chymotrypsin is that which is situated in close vicinity to the active site. If it is replaced by another amino acid, none of the remaining tryptophans react in the analogous enzyme elastase.

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