

# L-USnate-Urease Interactions: Binding Sites for Polymerization

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## Urease, L-USnic Acid

L-usnic acid inactivates urease through a process which implicates the blockade of –SH groups in parallel to the formation of inactive polymers. Both L-alanine and L-proline partially reverses the inactivation and effectively diminishes the amount of highly polymerized protein. The amino acids also prevent the linkage of L-usnic acid on the sites of low affinity for the ligand, being then related to the sites of polymerization.

## Introduction

L-usnic acid is powerful inactivator of native urease (molecular weight 480 000) through a process which implicates the formation of inactive aggregates with a molecular weight higher than that found for the native protein. The highest molecular weight of these polymers are 880 000 d [1] and 820 000 d [2] when they are formed *in vitro* conditions. *In vivo*, polymers of urease reach a molecular weight of 1 600 000 d [3], although other lichen phenols, as both chloroatranorin and evernic acid, can be related to the inactivation process.

The existence of two classes of binding sites for L-usnic acid on urease molecule has been proposed, the first showing high affinity for the ligand, related to the loss of activity, and the second, of low affinity, related to the polymerization process [4]. A similar way of interaction has been proposed for glucosamine-P isomerase (glutamine forming) [5]. 50 mM L-cysteine seems to reverse the inactivation of the highest molecular weight forms of urease [1] without any change of the polymerization degree. However, L-cysteine decelerates the co-operative binding of L-usnic acid on the high affinity sites without any modification of binding behaviour on the hypothetical low affinity sites [4].

Enzyme monomers probably associate by the formation of L-alanyl-L-usnate-L-prolyl bridges between different polypeptide chains [6]. This report is concerned with the aspects of the kinetic properties of L-usnate-urease interactions in order to clarify the formation of the highest molecular weight polymers of both inactive and active forms of the enzyme.

## Materials and Methods

Samples of 0.5 mg of crystalline urease (type III, Sigma Chemical Co.) were incubated at 37 °C for 5 min with 0.1 mM L-usnic acid in a final volume of 3.0 ml stabilized at pH 6.9 with 75 mM phosphate buffer. Immediately afterwards, the samples were assayed for enzyme activity using variable concentrations of substrate, according to the Conway microdiffusion method [7]. If indicated, 5, 15 or 30 mM both L-alanine and L-proline were included in the reaction mixture. Protein was estimated using the method of Warburg and Christian [8].

Separation of different aggregates was carried out by passing the samples through a Sepharose 4B column (21.0 cm in height × 3.0 cm in diameter) equilibrated with 75 mM phosphate buffer, pH 6.9. The main peaks of the filtrate were resolved by electrophoresis in 7.5 per cent acrylamide gel using the Fishbein method [9] and staining with Coomassie blue.

Binding experiments were conducted as previously described [4]. When L-alanine and L-proline were present, optical densities were corrected according to the absorbance values of the amino acids mixtures at 295 nm. Finally,  $\mu\text{mol}$  of bound ligand per  $\mu\text{mol}$  of protein were determined and Scatchard plots [10] were drawn.

## Results and Discussion

Preincubation of urease for 5 min, with 0.1 mM L-usnic acid produces about 60 per cent inactivation of the enzyme when urea concentration was 60 mM (Fig. 1). By including 30 mM L-alanine and L-proline in the incubation mixtures, a slight recovery of ac-

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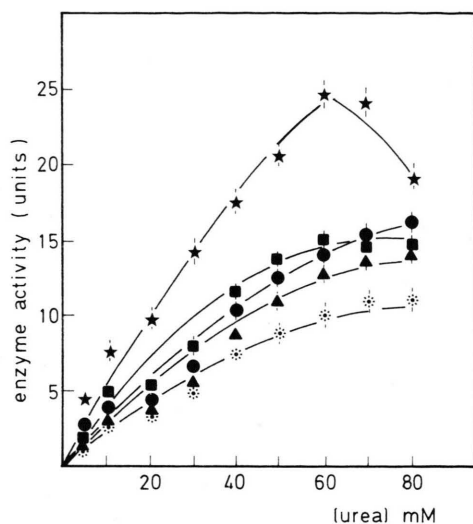


Fig. 1. Effect of both L-alanine and L-proline on the inactivation of urease by L-usnic acid. (\*) control without the drug; (○) both enzyme and inactivator preincubated before addition of substrate; (▲) 5 mM amino acids included during preincubation; (●) 15 mM amino acids and (■) 30 mM both L-alanine and L-proline included during preincubation. Values are the media of three replicates. Vertical bars give standard error.

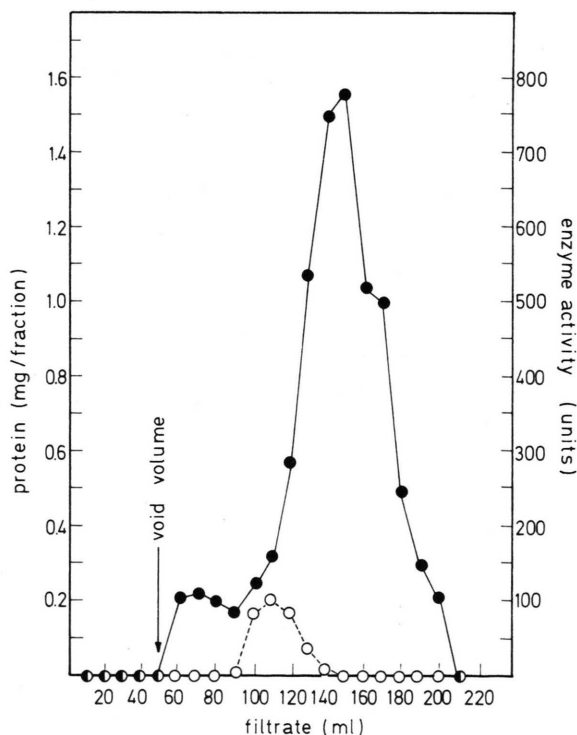


Fig. 2. Diagram of filtration on Sepharose 4B of 10 mg urease incubated for 5 min with 0.1 mM L-usnic acid. (●) mg protein; (○) specific activity.

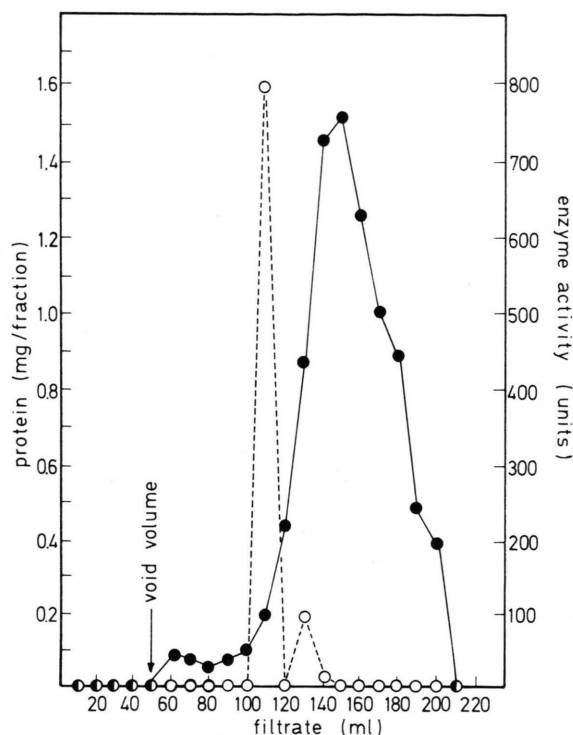


Fig. 3. Diagram of filtration on Sepharose 4B of 10 mg urease incubated for 5 min with 0.1 mM L-usnic acid in the presence of 30 mM both L-alanine and L-proline. (●) mg of protein; (○) specific activity.

tivity is observed since the loss of activity is reduced at a 50 per cent.

In order to analyze the action of the amino acids on the inactivation process, the incubation mixtures, containing both L-alanine and L-proline, are filtered through a Sepharose 4B column.  $\alpha$ -urease is recovered in the fraction which elutes at 150 ml of filtration (data are not shown). Inactive high molecular weight polymers produced by action of L-usnic acid elute at 60–90 ml of filtrate, these fractions being 9.36 per cent of the recovered protein. The major form of inactive urease elutes at 150 ml of filtrate, the enzyme which retains activity being displaced to an elution volume varying from 100 to 140 ml (Fig. 2). Similar pattern of elution is observed when 30 mM L-alanine and 30 mM L-proline are mixed with both enzyme and inactivator in the incubation mixture (Fig. 3) but, in this case, the inactive urease eluted at 60–90 ml of filtrate only represents about 37 per cent of total protein.

Inactive polymers are resolved as single bands in polyacrylamide gel electrophoresis. However, the

Table I. Active and inactive polymers of urease formed by action of L-usnic acid and both L-alanine and L-proline. The samples include 10 mg urease in a final volume of 10 ml. Incubations are performed as described in methods.

Treatment		Fraction from Sepharose 4B [ml]	Number of bands in acrylamide electrophoresis	Electrophoretic mobility
L-usnic acid [mM]	L-alanine + L-proline [mM]			
—	—	150	1	0.523
0.1	—	70	1	0.050
0.1	—	110	5	0.054 0.153 0.175 0.208 0.230
0.1	30	110	2	0.052 0.111
0.1	30	130	2	0.114 0.463

fractions which retain the highest values of activity show of very different behaviour. Fraction eluted at 110 ml after incubation with the inactivator is resolved in 5 different bands whereas the two peaks of active protein found when urease is incubated with the inactivator in the presence of both amino acids are resolved every one in two distinct bands, as shown in Table I.

Reversal of polymerization and recovery of activity produced by both L-alanine and L-proline can be related with the expected changes in the binding of L-usnic acid to the reversal by L-cysteine of the urease inactivation [4] by the drug. As shown in Fig. 4, L-usnic acid lies to the protein according to a biphasic curve which has been interpreted to indicate a limited number of binding sites of high affinity for the ligand (those that are protected by L-cysteine) together a large number of lower affinity sites [11]. The anomalous stabilization of high binding values has been interpreted [4] as a consequence of the low solubility of L-usnic acid in aqueous solvent [12, 13]. As a result, ligand saturation is not reached in the described experiments. By increasing the incubation time from 5 to 20 min, these results can be corrected. Fig. 5 shows a more conventional Scatchard plot that confirms the existence of two kind of sites for the ligand. Inclusion of both L-alanine and L-proline in the incubation mixtures decrease the relative values of binding L-usnate on the low affinity sites. Direct binding study support the conclusion that L-usnic acid bind less tightly to the enzyme incubated with both L-alanine and L-proline than it does to the untreated urease since the L-us-

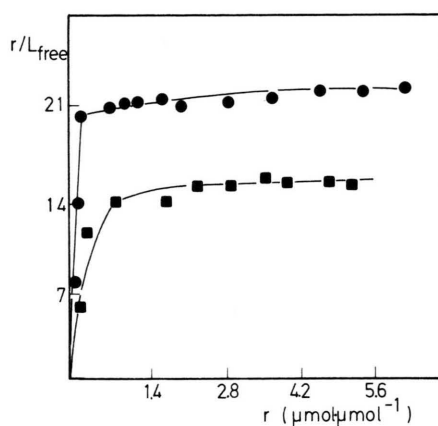


Fig. 4. Scatchard plots for binding of L-usnic acid on urease for 5 min incubation. (●) control with the drug and (■) 30 mM both L-alanine and L-proline.

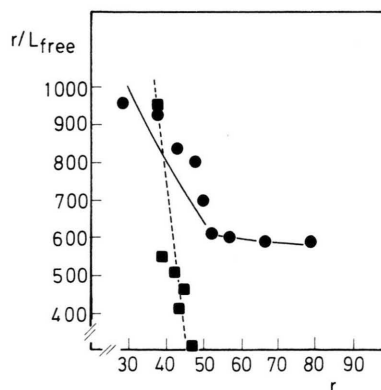


Fig. 5. Scatchard plots for binding of L-usnic acid on urease for 20 min incubation. (●) control with the drug, dissociation constant being  $0.102 \mu\text{M}$ ; (■) 30 mM both L-alanine and L-proline, dissociation constant being  $0.033 \mu\text{M}$ .

nate dissociation constant, determined according to Frieden and Colman [14] becomes larger, increasing about 3-fold when amino acids are absent from the incubation mixtures. Low affinity sites are, therefore, only related to the polymerization process. These facts could explain that the reversal of inactivation produced by both L-alanine and L-proline was lower than that achieved by L-cysteine [1] or

dithiothreitol [3] which protect the active site (high affinity site) of the enzyme.

#### *Acknowledgements*

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