The Mechanism of Deuterium Oxide-induced Protein Degradation in *Lemna minor*

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**Abstract.** Transfer of *Lemna minor* fronds to culture medium containing 50% (v/v) deuterium oxide induces a large increase in the rate of protein breakdown, which is not due to an increase in the activity of acidic or neutral proteolytic enzymes or peptidases. Biochemical and ultrastructural evidence indicates that deuterium oxide affects the properties of certain membranes, particularly the tonoplast, and allows vacuolar proteolytic enzymes to pass into the cytoplasm and cause the increased protein breakdown.

**Key words:** Deuterium oxide - *Lemna* - Protein degradation - Stress (D\(_2\)O) - Tonoplast properties

**Introduction**

Deuterium oxide (D\(_2\)O) has been extensively used for density-labelling experiments demonstrating de novo synthesis of enzymes and also to measure the half lives of individual enzymes (Johnson, 1977). However, we have shown (Cooke et al., 1979) that the transfer of fronds of *Lemna minor* to a medium containing 50% (v/v) D\(_2\)O leads to a loss of soluble protein from the fronds, due to a reduction in protein synthesis and a large increase in protein degradation.

The work described in this paper was undertaken to elucidate the possible mechanism of protein degradation brought about by the stress imposed by D\(_2\)O.

**Materials and Methods**

**Plant Material and Growth Media**

*Lemna minor* grown in sterile complete culture medium as described by Trewavas (1970) was used for all of the experiments. Where appropriate, the water in the medium was replaced by 50% (v/v) D\(_2\)O (Cooke et al., 1979). This is subsequently referred to as D\(_2\)O medium.

**Abbreviations:** BAPA = benzylarginine-p-nitroanilide; LPA = leucine-p-nitroanilide; TCA = trichloroacetic acid

**Estimation of Protein Degradation**

First order rate constants (kd) and half-lives (t\(_{1/2}\)) of total protein degradation were measured by a tritiated water (\(^3\)H\(_2\)O) technique (Humphrey and Davies, 1976). The 2\(^3\)H-content of hydrolysed protein amino acids was determined by racemisation with acetic anhydride.

**Assay of Proteinase and Peptidase Activity**

Proteolytic activity was measured by auto-digestion of extracted *Lemna* protein. Fronds (1.0–2.0 g fresh wt.) were frozen and ground up in 50 mM Tris buffer, pH 8.0 containing 3.5% (w/v) NaCl and 8 mM mercaptoethanol. The extracts were clarified by centrifuging at 20,000 g for 15 min, then de-salted by passage through Sephadex G-25 (12.0 x 3.5 cm columns). Reaction mixtures consisted of 1.0 ml of de-salted extract and 6.5 ml of either 25 mM Tris buffer, pH 7.1 or 25 mM citrate buffer, pH 5.2, both containing 8 mM mercaptoethanol. Mixtures were incubated at 37\(^\circ\)C for 2 h, after which 1.5 ml aliquots were removed, protein precipitated by the addition of 0.25 ml 40% (w/v) trichloroacetic acid (TCA) and the free amino acid content of the supernatants measured by a ninhydrin reaction. Proteinase activity is expressed as \(\mu\)moles amino released/g f.wt./h. It should be noted that these assays measure the proteolytic activity of the soluble fraction. There may well be proteolytic activity associated with the insoluble fraction.

Peptidase activity was measured as the p-nitroaniline released from leucine-p-nitroanilide (LPA) or benzylarginine-p-nitroanilide (BAPA) (Beevers, 1968). The assay mixture contained 0.5 ml de-salted *Lemna* extract and 0.9 ml 25 mM Tris buffer, pH 7.1 to which was added 0.1 ml 3 mM LPA or BAPA (dissolved in dimethyl formamide). The absorbance at 405 nm was measured initially and after 2 h incubation at 37\(^\circ\)C. Incubation was terminated by the addition of 0.5 ml 30% (w/v) acetic acid. Peptidase activity is expressed as \(\mu\)moles/g f.wt./h. In all experiments, total protein (as TCA-precipitable material) was determined by the method of Lowry et al. (1951).

**Electron Microscopy**

*Lemna* fronds, by virtue of their water repellent cuticle, are hard to fix for electron microscopy. The best results were obtained from fronds fixed in simultaneous glutaraldehyde and osmic acid made up in complete or stress medium as appropriate, following the method of Franke et al. (1969). Precooling of the plants made no observable difference and was therefore omitted. Fronds were cut under the surface of the fixative, post fixed in osmic acid, dehydrated in an acetone series and embedded either in Araldite or in Spurr’s resin. Thin sections were stained in uranyl acetate and lead citrate and examined in an AEI EM6B microscope.
Results and Discussion

Effect of $^2\text{H}_2\text{O}$ Medium on Proteinase and Peptidase Activity in L. minor

One possibility to account for stress-induced enhancement of protein breakdown would be that the activity of the proteolytic system is increased following transfer of Lemna fronds to $^2\text{H}_2\text{O}$ medium. Consequently, the proteinase activity at pH 5.2 and 7.1 and the peptidase activity against LPA and BAPA at pH 7.1 in Lemna fronds transferred for various periods of time to medium containing 50% $^2\text{H}_2\text{O}$ was measured. The results (Fig. 1) show that the activity of the proteinases and peptidases declines following the stress produced by $^2\text{H}_2\text{O}$ in a similar manner to the activity of several other enzymes that have been investigated (Cooke et al., 1979). Thus, increases in the activity of the proteolytic enzymes do not seem to be responsible for stress-induced enhancement of protein degradation. Consequently, we must consider an alternative mechanism.

Effect of $^2\text{H}_2\text{O}$ Medium on the Tonoplast

Assuming that the proteolytic system is located in the vacuole (see review by Matile, 1978), the increased rate of protein degradation brought about by transfer of Lemna fronds to stress medium could be due to changes in the permeability properties of the tonoplast. We have developed a technique (Davies et al., in preparation) to measure the partitioning of amino acids between the cytoplasm and the vacuole and have adapted this technique to investigate possible permeability changes occurring in the tonoplast when Lemna fronds are put under stress.

When Lemna fronds are transferred to a medium containing tritiated water ($^3\text{H}_2\text{O}$), the soluble amino acids become rapidly labelled at the C-2 position (Davies and Humphrey, 1976) due to exchange reactions catalysed by transaminases (Hilton et al., 1954). If labelled fronds are transferred to unlabelled medium, the transaminase catalysed exchange ensures that the $2^-\text{H}$ is rapidly lost from the amino acids. However, Fig. 2 shows that the $2^-\text{H}$ is not removed from the amino acids at a uniform rate, but rather suggests the existence of two pools of amino acids. One pool, which we assume to be cytosolic, loses its $2^-\text{H}$ rapidly, presumably due to the presence of transaminases and amino acids in the same compartment. The other pool, which we assume to be vacuolar, loses its $2^-\text{H}$ slowly, presumably due to the absence of transaminases from this compartment.

The results in Fig. 2 show that as the period of initial exposure to $^3\text{H}_2\text{O}$ is increased, the fraction of $2^-\text{H}$ slowly lost during the subsequent chase also increases. This is interpreted to mean that after a short exposure to $^3\text{H}_2\text{O}$, most of the $2^-\text{H}$ labelled amino acids are in the cytoplasm. As the time of exposure to $^3\text{H}_2\text{O}$ is increased, the amino acids in the vacuole become slowly labelled with $^3\text{H}$, presumably due to transport from the cytosol.

The effect of stress on this presumptive vacuolar amino acid pool was investigated. Lemna fronds were grown on complete medium containing $^3\text{H}_2\text{O}$ (3.8 x 10$^7$ Bq ml$^{-1}$) for 2 days and transferred to unlabelled complete medium for a further 2 days. This should ensure that only the vacuolar amino acid pool contains $2^-\text{H}$ (Fig. 2). The fronds were then placed on either unlabelled complete medium or unlabelled $^2\text{H}_2\text{O}$ medium, samples removed at intervals, the soluble amino acid fraction isolated (Humphrey and Davies, 1975) and the $2^-\text{H}$ content measured (Humphrey and Davies, 1976). Figure 3 shows that although there is a slight loss of $2^-\text{H}$ from the vacuolar amino acids of fronds maintained on complete medium, there is a much greater loss of $2^-\text{H}$ from the vacuolar amino acids of fronds transferred to the medium containing $^2\text{H}_2\text{O}$.

Thus, $^2\text{H}_2\text{O}$ increases the loss of $2^-\text{H}$ from what we have interpreted as the vacuolar amino acid pool. This indicates a stress-induced change in the properties of the vacuolar membrane, allowing an increased ef-