Measurement of Proteolytic Activity in Biological Materials using $^{125}$I-Labelled Casein

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The use of radioactively labelled proteins for studying proteolysis is well established (Schneider et al., 1965), as are techniques for radiolabelling proteins, which have been developed mainly for radioimmunoassay procedures (Yalow & Berson, 1966). $^{125}$I has been the favoured isotope for such work, although it has a short half-life (8.04 days). $^{125}$I has a longer half-life (60 days) and can be efficiently counted in the $^{14}$C channel of a liquid-scintillation counter.

The technique using $^{125}$I-labelled casein for studying proteolytic activity in microbiological materials, notably sewage and preparations of micro-organisms derived from it, is a simple one. After incubation of $^{125}$I-labelled casein with the materials, radiolabelled degradation products can be separated from the undegraded protein by perchloric acid precipitation. Subsequent use of a microcentrifuge enables each assay to be performed in a single reaction vessel on a total volume as little as 0.1 ml, minimizing the need for containment facilities for isotope or micro-organisms.

The assay suffers from few disadvantages compared with the alternatives available for these materials, such as gelatin penetration. Sewage-derived micro-organisms at a concentration of $10^6$/ml released sufficient radioactivity from the $^{125}$I-labelled casein in 90 min for measurement purposes. In theory, the sensitivity of such methods of enzyme assay is limited solely by the specific radioactivity of the radiolabelled substrate; in practice, due to self-irradiation, $^{125}$I-labelled casein undergoes decomposition leading to the release of acid-soluble radioactivity. The half-life of this process depends on the specific radioactivity; using a preparation of $^{125}$I-labelled casein prepared by the method of Hunter & Greenwood (1962) with the specific radioactivity initially of 10 Ci/g, the half-life of the bound radioactivity was similar to the isotope half-life, 63 days.


Hydrolysis of Artificial Substrates by Enterokinase and Trypsin and the Development of a Sensitive Specific Assay for Enterokinase in Serum

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The usual method for assaying enterokinase is by activation of its normal biological substrate, trypsinogen, and subsequently measuring the trypsin formed (Baratti et al., 1973). The disadvantage of this two-stage assay is that it precludes the sensitive determination of enterokinase in biological fluids such as serum, owing to the presence of trypsin inhibitors. The failure of these inhibitors to interact with enterokinase (unpublished work) should permit direct measurement of this enzyme. The activities of highly purified enterokinase and trypsin were tested against BzArgOEt,* BzArgNan and

* Abbreviations: BzArgOEt, $\alpha$-N-benzoyl-$\alpha$-arginine ethyl ester hydrochloride; BzArgNan, $\alpha$-N-benzoyl-DL-arginine p-nitroanilide hydrochloride; BzArgNHNap, $\alpha$-N-benzoyl-DL-arginine 2-naphthylamide hydrochloride.
BzArgNHNap together with a synthetic enterokinase substrate Gly-(Asp)_4-Lys-2-naphthylamide (Hesford et al., 1976). Gly-(Asp)_4-Lys-2-naphthylamide exploits the tetra-aspartic sequence characteristic of the activation peptides of mammalian trypsinogens, but since the susceptible band involves a lysine residue, it should, like trypsinogen, be cleaved by trypsin. In 25 mm-Tris/HCl, pH 8.4, containing 10 mm-Ca^{2+}, there was no detectable hydrolysis of BzArgOEt, BzArgNan or BzArgNHNap by enterokinase even after 16 h incubation at 37°C with 80 ng of pure enzyme. The kinetic parameters for trypsin were in close agreement with other workers (Table 1). With 1.5 mm-Gly(Asp)_4-Lys-2-naphthylamide as substrate, enterokinase activity increased in a hyperbolic fashion with increasing Ca^{2+} concentration up to 10 mm, and this was primarily due to a decrease in $K_m$ (Table 1). The response of trypsin to Ca^{2+} was sigmoidal and it was impossible to measure kinetic parameters. Under these assay conditions trypsin was found to be exceptionally labile if the concentration of Ca^{2+} was 2 mm or less. Colomb et al. (1978) have also shown the same instability for human trypsin at pH 8.0. Therefore the hydrolytic activity against Gly-(Asp)_4-Lys-2-naphthylamide was superimposed on the autolytic activity.

For both proteinases the amount of substrate hydrolysed was directly proportional to the quantity of enzyme incubated: 1.0–72 ng/ml for enterokinase and 0.5–1.0 μg/ml for trypsin. This was a 500-fold difference in terms of enzyme protein and represented an approximate 10^4-fold difference in molar amounts. Trasylol had no inhibitory action on enterokinase hydrolysis of Gly-(Asp)_4-Lys-2-naphthylamide up to a concentration of 2.5 μg/ml, an approximate molar excess of $5 \times 10^3$.

Rabbit anti-(human enterokinase) inhibits the activation of trypsinogen (Hermon-Taylor et al., 1977), but in this study it did not prevent hydrolysis of Gly-(Asp)_4-Lys-2-naphthylamide, even though controls confirmed total inhibition of trypsinogen-activating ability. This suggests that the antigenic determinants lie close to, but are not part of, the active site of enterokinase and so high-molecular-weight substrates are sterically prevented from binding at the active site.

Hydrolysis of Gly-(Asp)_4-Lys-2-naphthylamide by non-specific serum arylamidases was the main source of interference in the measurement of enterokinase which had been mixed with serum. This activity was completely inhibited by concentrations of Zn^{2+} higher than 3 mm, but no inhibition of enterokinase by this cation was observed. Endogenous serum trypsin concentrations are around 300 ng/ml in man and if this is so for the mouse it is well below the limits of detection in this system. α2-Macroglobulin-bound trypsin can hydrolyse low-molecular-weight substrates (Rinderknecht & Geokas, 1973), but no endogenous activity was found when Trasylol was omitted from a control tube. Therefore any hydrolysis under these conditions must be due to enterokinase alone and the calibration curve was in good agreement with that obtained in aqueous solution. The

### Table 1. Kinetic parameters for enterokinase and trypsin for four synthetic substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enterokinase</th>
<th>Trypsin</th>
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<tbody>
<tr>
<td></td>
<td>$K_m$ (mM)</td>
<td>$k_{cat.}$ (s⁻¹)</td>
</tr>
<tr>
<td>BzArgOEt</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>a</td>
</tr>
<tr>
<td>BzArgNHNap</td>
<td>a</td>
<td>a</td>
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<td></td>
<td>0.1</td>
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<tr>
<td></td>
<td>10.0</td>
<td>a</td>
</tr>
<tr>
<td>BzArgNan</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>a</td>
</tr>
<tr>
<td>Gly-(Asp)_4-Lys-2-naphthylamide</td>
<td>0.1</td>
<td>0.525</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.28</td>
</tr>
</tbody>
</table>

a, No hydrolysis detected; b, $K_m \geq 15$ mm; c, does not demonstrate simple kinetics.
presence of Zn$^{2+}$ and trasylol was not deleterious and only a 10% decrease in corresponding values was seen.


Inactivation of Sycamore Glutamate Dehydrogenase by Pyridoxal 5′-Phosphate

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Glutamate dehydrogenase (EC 1.4.1.3) from sycamore (Acer pseudoplatanus L.) cells grown in suspension culture has been purified to homogeneity and its molecular weight, subunit structure, amino acid composition and some kinetic properties determined. The mol.wt. is 270000, being composed of six subunits of mol.wt. 45000. Unlike glutamate dehydrogenase from bovine liver, no association into higher molecular weight aggregates occurs at enzyme concentrations up to 4 mg/ml. Like many glutamate dehydrogenases of plant origin (see e.g. Fawole & Boulter, 1977) both NADH and NADPH will serve as coenzyme, although NADH is preferred.

Chemical modification with reagents that interact specifically with certain amino acid residues has yielded valuable information on the involvement of such residues in the catalytic activity of many enzymes. Bovine liver glutamate dehydrogenase is substantially inactivated by the acetylation of one amino group per subunit (Colman & Frieden, 1966). Treatment with pyridoxal 5′-phosphate causes reversible inactivation resulting from modification of lysine-126 (Anderson et al., 1966; Piskiewicz et al., 1970). The NADP⁺-specific glutamate dehydrogenase from Neurospora crassa is also inactivated by treatment with pyridoxal 5′-phosphate (Blumenthal & Smith, 1973). Although inhibition by pyridoxal 5′-phosphate of partially purified pea epicotyl mitochondrial glutamate dehydrogenase has been described (Teixeira & Davies, 1974) no detailed study of the effect of this reagent on a purified glutamate dehydrogenase of plant origin has been published. In the present communication the effect of pyridoxal 5′-phosphate on sycamore glutamate dehydrogenase activity is described.

Fig. 1 shows the effect on glutamate dehydrogenase activity of incubation with various concentrations of pyridoxal 5′-phosphate. Incubations were carried out in the dark in 70 mm-sodium phosphate buffer, pH 7.5, at 25°C. At appropriate times samples were withdrawn and assayed spectrophotometrically in a standard assay. The reaction mixture (3 ml) contained 167 mM-NH$_4$Cl, 10 mM-α-oxoglutarate and 83 μM-NADH in 83 mM-sodium phosphate buffer, pH 7.5. Activity declined to a constant value that was dependent on the pyridoxal 5′-phosphate concentration. Increasing the pyridoxal 5′-phosphate concentration above 5 mM did not result in significantly increased inactivation. Addition of lysine (100-fold excess) during the incubation resulted in rapid reactivation. Inactivation was made irreversible by reduction with sodium borohydride.

Modification with 0.2 mM-pyridoxal 5′-phosphate for 60 min as described above, which resulted in the loss of 80% of the initial activity, was followed by reduction with

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