A recording viscometer for assaying mammalian collagenase

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A recording viscometer for monitoring the action of mammalian collagenase on soluble collagen is described. For this system, where only one peptide bond is cleaved per subunit, it is shown theoretically that the decrease in viscosity is proportional to the fraction of molecules cleaved. Experimental confirmation was obtained by parallel monitoring of hydrolysis by using the fluorescamine assay of liberated amino groups. The initial velocity of reaction is proportional to substrate concentration and enzyme concentration.

Viscometry has been used previously by several workers to assay collagenase and other hydrolases acting on high- M_r substrates (Evanson *et al.*, 1967; McCroskery *et al.*, 1975; Kuiper *et al.*, 1978; Salerno *et al.*, 1980). No apparatus appears to have been described, however, that monitors the progress of enzymic hydrolysis automatically and continuously, and a simple apparatus for achieving this is now described below. The change in viscosity has been correlated with the appearance of amino groups as assayed by the fluorescamine reaction (Schwabe, 1973) during the action of human skin collagenase on soluble collagen.

Experimental

Materials

Concentrated Dulbecco's modified Eagle's medium, Dulbecco's phosphate-buffered saline, Earle's balanced salt solution and foetal-calf serum were purchased from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. Other materials were purchased as follows: benzylpenicillin and streptomycin sulphate (Glaxo Laboratories, Greenford, Middx., U.K.), nystatin (E. R. Squibb and Son, London, U.K.) and bovine insulin (Sigma Chemical Co., Poole, Dorset, U.K.). Ile-Ala-GlyOEt was synthesized in this Department by Ms. G. Kay. Neutral-salt-soluble collagen was isolated from young rat skins by a modification (Jackson & Cleary, 1967) of the method of Gross *et al.* (1955).

Preparation of human skin collagenase

Fresh human skin obtained at surgery was grown as primary explants in culture by the method of Eisen *et al.* (1968). Alternatively, human skin fibroblasts were grown in culture as described by Bauer *et al.* (1975). Latent collagenase could be

activated either by trypsin or by trypsin that had been previously inactivated with Tos-Lys-CH₂Cl (Macartney, 1979). The collagenase was purified by the chromatographic procedures described by Woolley *et al.* (1978). The purified enzyme gave a single band on electrophoresis in a 7% polyacrylamide gel.

Spectrofluorimetric assay of collagen hydrolysis by collagenase

Amino groups, newly released from collagen by hydrolysis with collagenase, were assayed by the fluorescamine method of Schwabe (1973), with twice the quantities of reagents specified. A standard calibration curve was obtained with portions of a 100 μ M solution of Ile-Ala-GlyOEt in 0.2 M-sodium phosphate buffer, pH 6.8, with and without the addition of 0.2% (w/v) of neutral-salt-soluble collagen. The calibration curve was linear over the range 1.0–10.0 μ M of tripeptide ester; the addition of neutral-salt-soluble collagen made a negligible difference to the curve.

Viscometric assay of collagenase

The digest (4 ml) contained neutral-salt-soluble collagen (0.1–0.3%, w/v) and collagenase (1.7–8.7 μ g/ml) in 50 mM-Tris/HCl buffer, pH 7.4, containing 5 mM-CaCl₂; incubations were performed at 27°C. Smoothing of the data and computation of initial velocities were achieved by least-squares fitting to orthogonal polynomials (Elmore *et al.*, 1963).

Description of the recording viscometer

The layout of the viscometer is shown in Fig. 1. The solution of collagen and collagenase is pumped to the top of an Ostwald viscometer, and time of flow between two levels is measured and recorded automatically. This cycle of operations is repeated at

Fig. 1. Block diagram of the recording viscometer

Key: PT1, PT2 and PT3, phototransistors; PS1, PS2 and PS3, pulse shapers; FF1 and FF2, J-K 'flip-flops'; FS, fail-safe circuit to prevent operation of pump in the event of failure of either detector or its lamp.

appropriate constant intervals. The pump used is a standard heavy-duty aquarium pump connected so as to suck the solution containing enzyme and substrate from the reaction vessel to the top of the viscometer. The electro-valve connects the pump to the viscometer. Detection of the meniscus is achieved by shining rays of light from three lamp sources through the viscometer on to three phototransistors. On receipt of a timing signal from the interval timer (1–16 min), a J-K 'flip-flop', FF1, switches on both the pump and the electro-valve. Each phototransistor is switched off in turn as the meniscus passes and the light rays are diverted. When the top detector, PT1, is switched off, the output of 'flip-flop' FF1 is inhibited and the pump is switched off. Simultaneously the electro-valve is de-energized and the top of the viscometer is opened to the air. A fail-safe circuit is included to prevent the operation of the pump in the event of failure of either the detector or its lamp.

When the meniscus falls past the start-event time detector, PT2, the transistor is switched on, causing a pulse of 50ms to be transmitted from the pulse shaper, PS2. This pulse triggers the 'flip-flop' FF2. The inverted output $\bar{Q}$ is used to switch the event-time control circuit, which in turn enables an operational amplifier connected as a 'staircase generator' to step the chart recorder at 300ms steps. This output to the recorder (10mV full scale) can be stepped off at the three points to give full-scale deflexions of 1min, 2min and 5min. When the

meniscus passes the stop-event detector, PT3, the transistor is switched on and a 50ms pulse is transmitted from pulse shaper, PS3, and fed to the re-set pin, R_D , of the J-K 'flip-flop', FF2, causing the circuit to re-set. This re-sets the $\bar{Q}$ output and stops the recorder output from the 'staircase generator'. The pen returns to zero. The $\bar{Q}$ output from FF2 triggers a mono-stable circuit, which sends a drive pulse of 3s duration to the chart-drive motor of the recorder. This 3s burst of chart drive sets the pen up the chart for the next record of time of flow, which is initiated by a signal from the interval timers. The experimental record thus consists of a series of equidistant spikes, the height of each spike being proportional to the specific viscosity at that time. The viscometer and reaction vessel are enclosed in a glass-fronted cabinet maintained at a constant temperature. The radius of the viscometer tube was calculated after determining the weight of mercury it held at the working temperature. The accuracy of the viscometer was checked by measuring the dynamic viscosity of distilled water at various temperatures in the range 15–30°C. The values were within 1% of those in the literature (Kaye & Laby, 1968).

Theory

The action of collagenase on collagen can be followed on the recording viscometer as a function of time. A solution of partially hydrolysed collagen

is polydisperse, and so viscosity-average relative molecular masses must be used:

$$M_v = [\sum_i (N_i M_i^{1+\alpha}) / \sum_i (N_i M_i)]^{1/\alpha}$$

where M_i is the relative molecular mass of the i th species of which N_i molecules are present and α is a constant whose magnitude is dependent on molecular conformation. Collagenase cleaves collagen chains at a point approximately three-quarters of the distance from the N -terminus. The relative molecular masses of the fragments will be fM and $(1-f)M$, where M is the relative molecular mass of an intact collagen chain and f is approx. 3/4. Suppose that a fraction β of the N_0 original molecules have been hydrolysed. Then:

$$\sum_i N_i M_i = [\beta f M + \beta(1-f)M + (1-\beta)M] N_0 = M N_0$$

and

$$\begin{aligned} \sum_i N_i M_i^{1+\alpha} &= [\beta f^{1+\alpha} M^{1+\alpha} + \beta(1-f)^{1+\alpha} + \beta(1-f)^{1+\alpha} M^{1+\alpha} + (1-\beta)M^{1+\alpha}] N_0 \\ &= [\beta f^{1+\alpha} + \beta(1-f)^{1+\alpha} + (1-\beta)] M^{1+\alpha} N_0 \end{aligned}$$

This equation assumes that the value of α is unchanged by enzymic cleavage. Therefore:

$$M_v = [\beta f^{1+\alpha} + \beta(1-f)^{1+\alpha} + (1-\beta)]^{1/\alpha} M$$

At zero time ($\beta=0$):

$$\eta_0 = \eta_s + \eta_s K C M^\alpha$$

where η_s is the viscosity of solvent, C is the concentration of collagen and K is a constant. At time t :

$$\eta_t = \eta_s + \eta_s K C M_v^\alpha$$

Hence:

$$\eta_t = \eta_0 - \eta_s K C M^\alpha \beta [1 - f^{1+\alpha} - (1-f)^{1+\alpha}] \quad (1)$$

and the viscosity is proportional to β . But the flow time, τ , in an Ostwald viscometer is proportional to the viscosity. Hence $\tau_0 - \tau_t$ is proportional to β . This relationship only holds if cleavage occurs at a single site. Moreover, the increase in fluorescence resulting from the reaction of fluorescamine with the collagenase digest of collagen is proportional to β . Hence a linear relationship should exist between η_t and the observed fluorescence, F_t .

Results and discussion

The recording viscometer has been reliable and has given reproducible results in replicate kinetic runs. It has chiefly been used to assay preparations of human collagenase from normal and malignant tissue and to assess the inhibitory properties of

synthetic peptides. It could obviously be used to monitor the activity of other hydrolases such as hyaluronidase, pectinase, amylases and nucleases.

Under the conditions used here (27°C, pH 7.4), the viscosity is a non-linear function of time. The fragments produced by the action of collagenase on collagen are not thermally denatured (McCroskery *et al.*, 1975), and it is therefore probable that α is not appreciably changed.

The rate of cleavage of collagen by collagenase as measured by the fluorescamine method is also a non-linear function of time. In contrast, there is a linear relationship between viscosity and release of new N -terminal residues, as required by eqn. (1) (correlation coefficient $r=0.9993$ in a typical run). In contrast, the corresponding plot with the bacterial collagenase from *Clostridium histolyticum* was

non-linear. This result was expected, since the enzyme cleaves collagen at several sites.

Since the rate of increase in fluorescence in the fluorescamine assay is proportional to the rate of hydrolysis, the rate of decrease in viscosity is also proportional to the rate of hydrolysis.

The initial velocity of reaction measured viscometrically was proportional to the substrate concentration ($r=0.9980$). Consequently, the highest substrate concentration used (0.3%, w/v) is considerably lower than K_m . In addition, the initial velocity of reaction was proportional to the concentration of collagenase ($r=0.9918$).

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