

Starch-Gel Electrophoretic Pattern of Pepsin-Treated Collagen

R. PENTTINEN, A. KARI and E. KULONEN

Department of Medical Chemistry, University of Turku, Turku 3, Finland

When acetic acid-soluble rat-tail-tendon collagen was digested with pepsin, at first the β -components and larger aggregates were broken down to α -components. In the second phase there were liberated at least seven distinct fragments, which yielded a reproducible starch-gel electrophoretic pattern. The effects of the various conditions during the digestion are described.

Pepsin (EC 3,4,4,1) is known to solubilize insoluble collagen,¹ *e.g.*, from cornea,² and to change the reconstitution properties of collagen, presumably by detaching so-called telopeptides, which are assumed important in the organization of collagen.³⁻⁵ Other authors claim that pepsin affects the non-collagenous impurities, attached to collagen.^{6,7} The released peptides are especially rich in tyrosine.⁸ The denatured collagen or gelatin is degraded by pepsin to fragments with alanine and valine as the terminal amino acids.⁹

Since the "telopeptides" have been assigned important roles in the formation of the intra- or intermolecular linkages between the peptide chains, we wished to study the effect of pepsin on the starch-gel electrophoretic pattern of components in collagen in various conditions, also above the transition temperature. The second purpose was to achieve a limited proteolysis of collagen.

EXPERIMENTAL

Preparation of collagen samples. Rat-tail tendons of adult albino rats were dissected in the cold room with forceps, rinsed with cold water and blotted dry. The material was extracted with 100-fold amount (v/fresh weight) of 3% acetic acid (w/v) for 36 h. The solution was centrifuged in the MSE refrigerated centrifuge at 35 000 *g* for 150 min. The supernatant was dialyzed in the cold against 0.5% (w/v) acetic acid for 48 h and centrifuged again. The supernatant was lyophilized and the dry residue stored at +4°C in a vacuum exsiccator. The samples were dissolved into 0.5% (w/v) acetic acid by shaking for 24 h. The reference samples were dialyzed against pH 4.7 acetate buffer, ionic strength 0.017, and stored frozen. The concentrations of collagen in the solutions were about 0.5% (w/v) in most experiments.

Treatment with pepsin. Pepsin was prepared by E. Merck AG, Darmstadt, Germany (Pepsinum DAB 6, 1:3500, Lot No. 74531). It was dissolved into 0.5–2.0 N hydrochloric acid.

For standard experiments the collagen solution in acetic acid was brought to pH 1.5–2.0 by addition of 0.5–2.0 N hydrochloric acid. The necessary amount of pepsin solution was added and the respective amount of hydrochloric acid was added to the control sample. Enzyme:substrate ratios from 1:10 to 1:200 (w/w) were tried and the ratio of 1:100 was selected for standard use. As a rule, the digestion was allowed to proceed for 3–6 h at +25°C, but in some experiments the digestion was carried out at +40°C. This procedure was modified according to the purposes of each experimental series.

The digests were kept frozen until the electrophoretic fractionation. All the samples were denatured immediately before each electrophoretic run for 15 min in a +40°C water bath.

Electrophoresis. The starch-gel electrophoresis was carried out under optimal conditions¹⁰ in 110 mm broad gel sheets. The gel concentration was 14.7 % (w/v) of hydrolyzed starch (Connaught Research Laboratories Ltd., Toronto, Canada) in acetate buffer, pH 4.7, ionic strength 0.017, saturated with octanol. In one series buffers of varying pH were used. A voltage of 120 V (gradient about 7.0 V/cm) was used and the run lasted regularly 6 h. The staining of the sheets with nigrosin has been described separately.¹⁰

Various modifications in the electrophoretic procedure were tried to facilitate the separation of the bands, varying pH of the electrophoresis buffer, ionic strength and gel concentration, but no improvement was observed.

Rubin *et al.*⁵ reported that in the present pH range pepsin is partially autolyzed yielding some small peptides. In our experiments pepsin moves to the anode and a colourless area, due to pepsin, is observed in the bluish nigrosine-stained gel sheet. No bands in the cathodal direction could be demonstrated in control experiments, when pepsin alone was incubated without collagen.

As a rule, the digests were not neutralized before the imbibition to the paper strips, which are inserted into the gel at the beginning of the run.¹⁰ The acidity is rapidly neutralized at the beginning of the run as ascertained with gel sheets, which were stained with suitable indicator dyes after the electrophoretic run.

RESULTS

Duration of the treatment with pepsin. The effect of pepsin on the starch-gel-electrophoretic pattern of denatured soluble collagen is shown in Fig. 1. The β -subunits and the x -units (larger aggregates) disappear even after a short digestion (30 min) at +25°C, but the α -components remain in the digest for relatively long time. There appear at least seven distinct bands (A-G) which migrate faster than the α -components.

Samples were taken at various intervals (from 15 min to 48 h) after the addition of pepsin and the digestion was stopped by freezing. No changes were observed in the patterns of those samples which had been incubated for the same time at pH 2.0 without pepsin. The effect of the increased digestion time appears in the increased staining intensities, especially of the two fastest bands, which remain constant after the digestion for about 3 h. After a long digestion (24 h or more) at +40°C the α -components disappear almost entirely and the bands become blurred. In those circumstances even the control samples begin to decompose.¹¹

Effect of pH during the digestion with pepsin. Collagen samples were dissolved in acetate buffer of varying pH and the hydrogen ion concentrations below pH 4.0 were obtained by addition of N hydrochloric acid. Pepsin was in this experiment dissolved into 3 % acetic acid. The digestion was carried out at

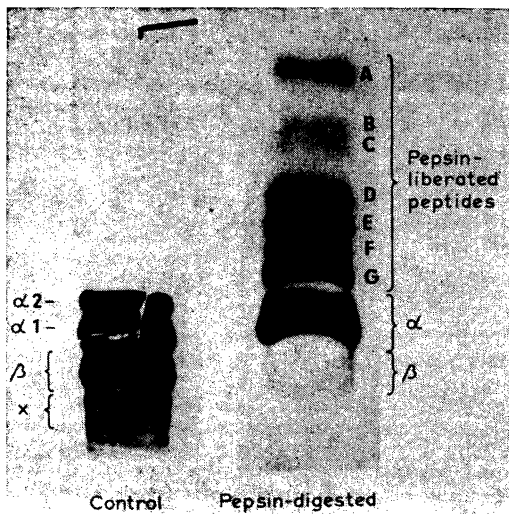


Fig. 1. Effect of the digestion with pepsin on the starch-gel electrophoretic pattern of rat-tail-tendon collagen. The control is the same sample, incubated but without pepsin. Pepsin:collagen 1:100, acetate-HCl buffer, pH about 2.0, temperature +25°C, duration 3 h. The electrophoresis was carried out in standard conditions¹⁰ after heat-denaturation.

+25°C for 3 h and the electrophoresis at standard conditions¹⁰ (pH always 4.7). The effect of pepsin was apparent in the pH range 1.8–4.5, and optimal at pH 1.8–2.8. The electrophoretic pattern depends on pH during the digestion, suggesting separate proteolytic actions.

In a series of experiments it was studied how the migration patterns of the pepsin-liberated fragments depend on the pH during the electrophoretic run. The pattern did not change more than expected from earlier work.¹⁰

Effect of temperature. Three experimental series were done, each comprising the temperature range of +15° to +40°C. The first two series were carried out with 1 % (w/v) collagen (viscous gel) and the third with a 0.5 % collagen solution. The results were somewhat variable. When 0.5 % collagen was used as a substrate, the temperature +25°C already was sufficient for an effect of pepsin.

The viscous 1 % collagen was not degraded when digested below +30°C (Fig. 2). It should be noticed that all the samples were denatured for 15 min at +40°C in the presence of pepsin before the electrophoresis and that some enzymic degradation continues during this period. Control experiments ascertain that in the present conditions the time intervals from the denaturation to the start of the electrophoresis are not critical.

The addition of hydrochloric acid to obtain the desired pH at digestion decreases the denaturation temperature of collagen.¹²

Dialysis of the pepsin-digest of collagen. When the digestion mixtures were dialyzed against acetate buffer, pH 4.7, ionic strength 0.017, at +4°C

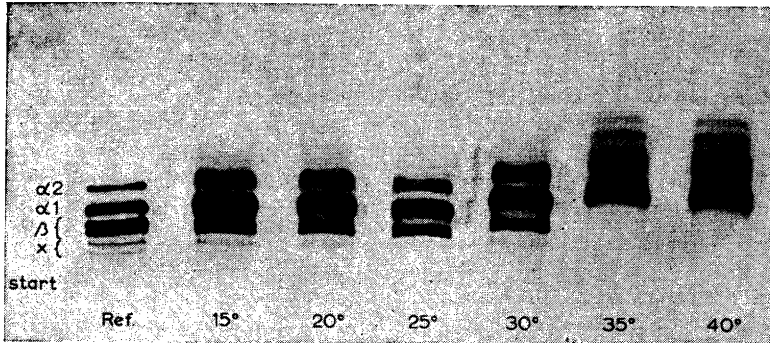


Fig. 2. Effect of temperature during the digestion with pepsin on the starch-gel electrophoretic pattern of rat-tail-tendon collagen. The reference is the same sample, non-incubated and without addition of pepsin. Pepsin:collagen 1:100, pH of HCl-acetate buffer about 2.0, duration 3 h, concentration of collagen 1%. The electrophoresis was carried out in standard conditions¹⁰ after further heat-denaturation.

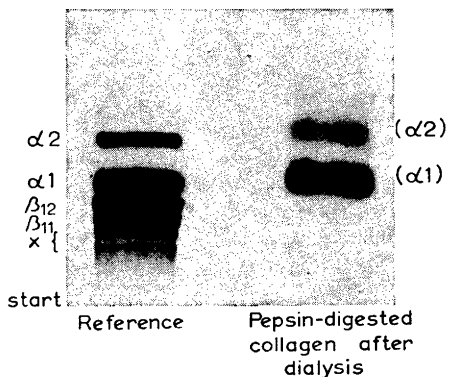
for 48 h and the non-dialyzable fraction studied by electrophoresis, the fast bands almost disappeared but the bands corresponding to α -components were still present. After long dialysis in the cold room at $+4^{\circ}\text{C}$ (Fig. 3) the fast bands disappeared entirely and only the two main bands corresponding to $\alpha 2$ and $\alpha 1$ and a weak fraction (corresponding to F in Ref. 11) remained.

DISCUSSION

In the present experiments we obviously deal with the hydrolysis of denatured collagen by pepsin. The β -components and larger aggregates disappear also after the treatment of non-denatured collagen with pepsin as shown by Rubin *et al.*⁴

It is not yet possible to state whether the bands migrating with the rate of the $\alpha 1$ - and $\alpha 2$ -components are actually the original peptide chains or a

Fig. 3. Effect of prolonged dialysis on the starch-gel electrophoretic pattern of pepsin-digested collagen (*cf.* Fig. 1). The reference is the same sample, non-incubated and without addition of pepsin. Pepsin:collagen 1:100, acetate-HCl buffer, pH about 2.0, temperature $+40^{\circ}\text{C}$, duration of digestion 7 days. Duration of dialysis 1 month; outside fluid acetate buffer, pH 4.7, ionic strength 0.017, saturated with octanol, changed weekly; temperature $+4^{\circ}\text{C}$. The electrophoresis was carried out in standard conditions¹⁰ after heat-denaturation.



kind of "de-tailed collagen". The other liberated fragments, (A-G) cannot all be very small, because they do not diffuse off from the gel sheets during the staining procedure. Courts⁹ found the number average molecular weight of 10 500 in pepsin-hydrolyzed gelatin.

Some other proteolytic enzymes, *e.g.* papain, ficin, trypsin, and chymotrypsin, were also tested. The results were unsatisfactory, because the enzymes migrated in the electrophoresis in the same direction as the breakdown-products from denatured collagen and the bands were not distinct (Lampiaho, unpublished work).

This procedure offers an opportunity to prepare α -like units or to cleave denatured collagen to a simple mixture of degradation products. Work is in progress for the preparative isolation of the fragments, but thus far no single method has been usable for a complete fractionation of these fragments.

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