The Use of Ethylene Maleic Anhydride for the Preparation of a Water-soluble Polyanionic Derivative of Pepsin. Preparation and Properties

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The pH-activity profile for several enzymes covalently bound to polyanionic or polycationic carriers, may be shifted towards alkaline or acidic pH values, respectively, when compared with the corresponding profile of the native enzyme.[1] It has been shown that the pH-activity profile of pepsin (EC 3.4.23.1) may also be shifted, when coupled to polyelectrolytes as glass and probably agarose and cellulose.[2-4] However, all these modifications give rise to insoluble derivatives. Furthermore, most of the coupling reactions proceed at optimum rate at alkaline pH, where pepsin is rapidly and irreversibly denatured. Ethylene maleic anhydride copolymer (EMA) which is an example of a polyanion may be coupled to proteins [5,6] even at pH values lower than 6.[7] The aim of this work was to determine whether porcine pepsin covalently bound to a soluble polyanionic carrier could be used to catalyse the clotting of milk in the pH range 6 – 6.5 where native porcine pepsin is rapidly inactivated.

In the present paper the preparation of a catalytically active and watersoluble polyanionic enzyme (EMA-pepsin) is described. The coupling was undertaken at pH 5.8. The activity against macromolecular substrates (Haemoglobin and reconstituted skim milk) of EMA-pepsin was measured. The change in activity relative to native pepsin reflected the influence of the electrostatic field, induced by the attached polyelectrolyte chain. A marked enchancement of stability in the pH range above pH 6 was demonstrated in the EMA-modified pepsin.

Pepsin (2 × crystallized porcine pepsin) was purchased from Worthington Biochem. Corp., Freehold, N.Y., U.S.A. Bovine haemoglobin (cryodialyzed) was a gift from Børge Thing, Finseninstitutet, Copenhagen, Denmark. Nonfat low-heat spray-dried milk powder was from West Comp., Fon du Lac, Wisc., U.S.A. Chymosin (Rennin) was a gift from Chr. Hansen's Laboratorium Ltd., Copenhagen, Denmark. Linear ethylene maleic anhydride copolymer (EMA grade 11) was generously provided by Monsanta Ltd., La Salle, Quebec, Canada. The molecular weight was about 20 000 Dalton as determined by sedimentation equilibrium in the ultracentrifuge.[7] Formaldehyde (35 % W/W solution) and sodium borohydride was from Merck, Darmstadt, Germany. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was from Sigma, St. Louis, U.S.A.

200 mg pepsin was dissolved and 400 mg EMA 11 was suspended in 20 ml 0.05 M sodium phosphate buffer pH 5.8. The reaction was carried out at 30 °C for about 20 h in a pH-stat, equipped with a thermostated reaction vessel (25 ml) (Radiometer, Copenhagen, Denmark). During the reaction the pH was kept constantly at 5.8 by addition of 2 M sodium hydroxide. EMA-pepsin and EMA were precipitated with dilute hydrochloric acid (final pH 2.2) and ultracentrifuged for 20 min at 17 000 *g*. The centrifugation was repeated 4 times with intermittent washing of the precipitate with potassium chloride/hydrochloric acid buffer pH 2.2, ionic strength 0.1. Finally the precipitate was redissolved in 0.5 M sodium phosphate, and the pH was adjusted to 5.8. The modified enzyme was stored at −20 °C. The final volume was 10 ml.

Determination of pepsin contents in the precipitate and the supernatants was performed by amino acid analysis [9] for aspartic and glumatic acids, threonine, serine, glycine, and valine and comparison with the contents of these amino acids found in a pepsin solution with known concentration.

The average number of EMA-molecules bound per pepsin molecule was calculated from the number of residual free amino groups. These were determined by the TNBS method according to Fields [8] and by amino acid analysis [9] after reductive methylation with formaldehyde and sodium borohydride according to Means and Feeney.[10]

The rate of proteolysis of reconstituted milk was determined as described by Foltmann [11] with the following modification: Ten ml of reconstituted skim milk and one ml of 0.05 M sodium phosphate buffer pH 6.0, 6.3, or 6.5 were placed in one branch and the enzyme solution (not more than 100 µl) in the other branch of a bifurcated glass tube. Reconstituted milk with pH-values of 6.0, 6.3, and 6.5 at 20 °C was obtained by dissolving the dry skim milk powder in 0.04, 0.01, and 0.001 M calcium chloride, respectively.

For stability measurements, the enzyme was stored in 0.05 M phosphate buffer of pH 6.3 and 6.5 at 30 °C, for varying length of time before determination of the proteolytic activity. The time of coagulation was in all cases between 4 and 5 min.

Measurements of the proteolysis of haemoglobin was carried out according to Kassell and Meitner [12] using 1.25 % haemoglobin solutions, ionic strength 1.0 and 0.010 and pH between 4 and 6, as substrates. Agarose gel electrophoresis was performed according to Ref. 13.

Coupling of EMA to pepsin by the method described here gave yields of about 30 %. Estimates of the yield based on the different amino acids were in good agreement and the

method was reproducible. (Coupling experiments performed at low temperature in acetone-water mixtures according to Centeno,[6] but at pH 5.8 in stead of 7 showed only negligible conjugation). In dilute HCl the EMA-pepsin and free EMA were selectively precipitated, leaving unmodified pepsin in the supernatant. The recovery of EMA-pepsin was 95 % of the yield after four washings and the amount of pepsin in the last wash less than 2 % of the amount in the initial reaction mixture. Isolated EMA-pepsin was stable during storage at 5 °C for at least two months at pH 5.8, and ionic strength about 1.0. Agarose gel electrophoresis of the reaction mixture showed two distinct protein bands, whereas pepsin only gave one.

The pepsin molecule contains only one ε-amino group and one α-amino group. Amino acid analysis after reductive methylation of EMA-pepsin was performed. It was demonstrated that nearly 100 % of the lysine was modified. Modification of the residual free N-terminal α-amino group in pepsin cannot be detected by this method. However, a determination of the residual free primary amino groups of the isolated EMA-pepsin using the TNBS method showed that 50 % of these groups were modified. This makes it reasonable to suggest that mainly lysine was modified with EMA under the selected conditions. Binding of two or more pepsin molecules to the same EMA molecule might be excluded as the agarose gel electrophoresis only showed two bands.

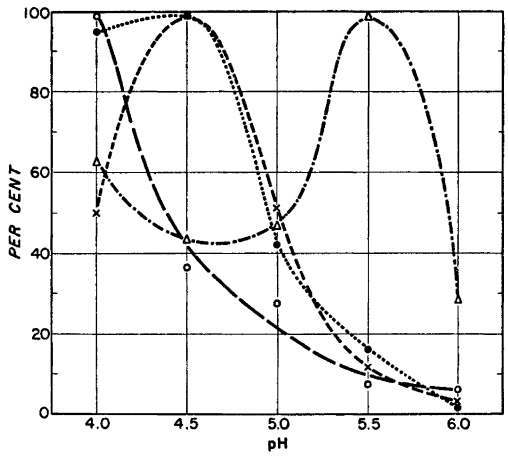

Fig. 1. pH-Activity profiles for pepsin and EMA-pepsin measured at different ionic strength using haemoglobin as substrate. The individual activities were calculated as the mean of four measurements (range ± 5 to 15 %) and expressed as the percentage of the activity at pH-optimum. ●, pepsin ($\Gamma/2 = 1.0$); ○, pepsin ($\Gamma/2 = 0.010$); ×, EMA-pepsin ($\Gamma/2 = 1.0$), △, EMA-pepsin ($\Gamma/2 = 0.010$).

pH-activity profiles of pepsin and EMA-pepsin measured in the pH-range 4 – 6 using haemoglobin as substrates are shown in Fig. 1. The profiles of the polyanionic derivatives of pepsin are displaced towards more alkaline pH-values by 1.5 pH units at low ionic strength ($\Gamma/2 = 0.010$) as compared with the native enzyme. The displacements are less pronounced at higher ionic strength ($\Gamma/2 = 1.0$). The enzymatic activity at 30 °C is expressed as percentage of the maximum activity obtained. The pH-activity profile shift could be attributed to a lowered intrinsic pH arising from a polyanion near the domain of the active site of the enzyme. The theoretical aspects of the electrostatical field prevailing in the microenvironment of polyanionic enzymes are discussed in several papers.[1,14] Another explanation of the pH-shift might be decreasing solubility of EMA-pepsin at pH below 4. However, turbidometric measurement shows that EMA-pepsin is soluble even at pH 3, ionic strength 0.01 and 1, respectively.

Comparison between the activity of pepsin and EMA-pepsin against reconstituted milk is shown in Table 1. The low activity of EMA-pepsin compared with that of pepsin may reflect steric hindrance due to the bulky EMA-group which will reduce the contact between the active centre of the enzyme and a macromolecular substrate. Also interaction between

Table 1. Proteolytic activity of EMA-pepsin relative to unmodified pepsin at different pH-values measured on reconstituted milk.[11]

pH	% Activity
6.0	8.2 (±.7)
6.3	11.6 (±.1)
6.5	14.8 (±.05)

Table 2. Half-lives for proteolytic activity of pepsin and EMA-pepsin at pH 6.3 and 6.5. Half-lives were calculated from the time dependent decrease in proteolytic activity against reconstituted milk. The activity was defined to be 100 % at time zero, and the kinetics of inactivation to be of first order. The coefficient of the correlation found by first order kinetic plots are given in brackets.

pH	Pepsin $t_{1/2}$(min)	EMA-pepsin $t_{1/2}$(min)
6.3	70 (−0.98)	180 (−0.95)
6.5	30 (−1.00)	150 (−1.00)

positively charged substrate groups and EMA might prevent catalysis.

The stability of pepsin and EMA-pepsin in the pH-range above 6.0 was determined using calf chymosin as internal standard. The activity was measured relatively to pepsin at 30 °C. The results are given in Table 2 as half-lives. It is seen that the stability against the irreversible denaturation is three–five times better for EMA-pepsin than for unmodified pepsin.

For pepsin an electrostatic expansion of the negatively charged polypeptide chain may lead to denaturation at pH-values higher than 6. The enchanced stability of EMA-pepsin relative to pepsin may be attributed to different micro-environmental states of the two enzymes.

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Tritium—Protium Exchange in Dextran at 25 °C

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In the dextran (Sephadex®) gels the cross-links are connected by ether linkages to the anhydroglucose residues of the dextran chains. Thus, at each point of attachment of a cross-link an hydroxyl hydrogen is lost and the concentration of hydroxyl groups remaining after cross-linking provides valuable information about the cross-linking structure. Since hydroxyl hydrogens will exchange with deuterium (D) or tritium (T) [1] provided they are accessible, tritium exchange can, thus, be used, giving information about the fraction of accessible groups, to determine the hydroxyl concentration.

The degree of accessibility of the Sephadex hydroxyls is, as yet, unknown. As regards other polysaccharides all the hydroxyls of amylose and starch, notwithstanding some degree of crystallinity in the latter, were reported to exchange with deuterium of deuterated water.[2] The 1,6-linked polysaccharides, *e.g.* dextran, are conformationally very flexible with an extended structure [3] and inaccessibility is unlikely but should be tested for. The lack of crystallinity in a dry sample of the most highly cross-linked Sephadex gel,[4] G-10, while not proof of accessibility, is strongly suggestive of it.

This communication reports an attempt to measure the tritium exchange constant of the parent polymer dextran as a model for the dextran gels. The exchange reaction between tritiated water (HTO) and the gel hydroxyls is

$$\mathrm{HTO} + \mathrm{Gel-OH} \rightleftharpoons \mathrm{Gel-OT} + \mathrm{H_2O}$$

Because of the relatively large difference in their vibrational zero point energies,[5,6] the bond dissociation energies of the different hydrogen isotopes differ sufficiently for the exchange constants to deviate considerably from unity in many systems; the T/H value usually diverging more than the D/H.

Experimental. Dextran® ($\overline{M}_w = 250\,000$; $\overline{M}_n = 113\,000$) was dissolved, without predrying, in double quartz-distilled water to a concentration of approximately 40 %. The concentration (weight fraction of dextran = 0.3604) was then calculated from the polarimetry [7] (Na D line 589 nm) of triplicate gravimetric dilutions (~2 % solutions).

About 15 ml of the 36 % dextran solution were placed in one compartment of a double glass flange (FG 25, Quickfit, Great Britain) chamber and were separated from 15 ml water containing HTO (1 mCi/ml) in the other compartment by a hydrophobic polyvinylidene