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Characterization of Papaya Peptidase A as an Enzyme of Extreme Basicity

NIELS C. KAARSHOLM and POUL SCHACK *

Fysisk-Kemisk Institut, DTH 206, The Technical University of Denmark, DK-2800 Lyngby, Denmark

Papaya peptidase A, a papain-like enzyme, has formerly been shown to contain a larger excess of basic amino acids and to have a higher isoelectric point than any of the other enzymes in the papaya latex. Determinations of the free electrophoretic mobility as a function of pH now establishes the isoelectric point of papaya peptidase A as 11.7 and that of succinylated papaya peptidase A as 3.8. Although the specific activity of the enzyme appears only slightly affected by the succinylation, the accompanying change in the charge/mobility ratio seems to indicate a relatively large conformational change upon succinylation.

Papaya peptidase A [1] is a proteolytic enzyme of the latex of *Carica papaya* very similar to papain (EC 3.4.22.2). Among the few differences revealed by comparison of the two enzymes, a prominent one is the high content of lysine in papaya peptidase A compared with that of papain. Whereas papain is known to possess 10 lysine residues [2] a number of 22–23 has been reported for papaya peptidase A.[1,3] Due to its extreme basicity the isoelectric point of papaya peptidase A has never been exactly determined. Schack [1] estimated an isoelectric point of "near 11" using free electrophoresis, I=0.1 M, whereas Brocklehurst *et al.*[4] claimed "above 11" for the protein prepared from spray dried latex and a value of 10.9 for other preparations of the enzyme. In the latter cases, however, neither the method nor the ionic strength were stated.

We here report electrophoretic mobility determinations at constant current for papaya peptidase A in the pH-range 9.8–12.4 using glycine/NaOH buffers, I=0.14 M. This reveals an isoelectric point of 11.7 for papaya peptidase A, to our knowledge the highest value reported for any protein.

Furthermore, it is demonstrated that succinylation of papaya peptidase A is associated with a loss of at least 20 free amino groups and a concomitant decrease in isoelectric point of approximately 8 pH units without any decrease in specific activity towards *N*-benzoyl-L-arginine ethyl ester (BAEE) at pH 6.0.

EXPERIMENTAL

A lyophilized extract of commercial dried papaya latex (Sigma, crude type I, lot 127C-0334) was prepared according to Schack.[1] From this material papaya peptidase A was prepared by means of a batchwise procedure: 5 g lyophilized extract dissolved in 100 ml 0.4 M NaCl was equilibrated with 50 ml CM-Sepharose CL-6B slurry (Pharmacia, Sweden). The suspension was transferred to a sintered glass filter and washed with 500 ml 0.4 M NaCl added in several portions. Papaya peptidase A was subsequently eluted by washing with 2×50 ml 1.2 M NaCl, the fractions of which were pooled, dialyzed and lyophilized according to Schack.[1] The preparations thus obtained appeared homogeneous by the criteria of disc-electrophoresis and ion-exchange chromatography. Upon activation with dithiothreitol the preparation exhibited a thiol content of typically 0.35 mol of SH per mol of protein as determined by titration with 2,2′-dipyridyl disulfide.[5]

* To whom correspondence should be addressed.

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Succinylation of the unactivated enzyme was performed according to the method of Sluyterman and De Graaf [6] for the succinylation of papain. The modified enzyme was stored lyophilized.

Cytochrome *c* type VI from horse heart was the product of Sigma. Free electrophoretic mobilities were determined by zone electrophoresis using the constant current method of Waldmann-Meyer.[7] Whatman 3 MM paper was used as the carrier and the following buffers were employed: pH 3.7–5.0 sodium acetate–acetic acid, pH 6.0–7.0 sodium barbiturate–acetic acid, pH 9.8–12.4 glycine–NaOH. The electrophoretic experiments were generally performed using 3 % (w/v) solutions of lyophilized protein in the respective buffers. In the case of papaya peptidase A control experiments were run employing the mercury derivative of fully active papaya peptidase A which was prepared by covalent chromatography using Thiopropyl-Sepharose 4B (Pharmacia, Sweden) and a method similar to that of Stuchbury *et al.*[8] for the preparation of papain. The pH dependence shown by the electrophoretic mobilities obtained for the mercury derivative of the fully active enzyme was indistinguishable from that obtained for only partly active preparations of the enzyme.

Free amino groups were determined by a modification of the trinitrobenzenesulfonic acid (TNBS) method of Snyder and Sobocinski [9] using $NaHCO_3$–Na_2CO_3 buffer, pH 9.3. Glycine was employed as reference. *O*-succinylation of hydroxyamino acids (serine and threonine) was determined by means of the alkaline hydroxylamine reaction of Hestrin.[10] Succinic monomethylester (a generous gift from the Department of Organic Chemistry, this University) was used as standard. Esterase activity towards *N*-benzoyl-L-arginine ethyl ester (BAEE, Sigma) at pH 6.0 was determined using a pH-stat (pH-meter 28/autoburette ABU 11/titrator TTT 11, Radiometer, Copenhagen). The concentrations of enzyme and substrate were typically 1 μM and 5 mM, respectively. Assay mixtures contained 10^{-4} M dithiothreitol. Specific activity was expressed as initial velocity divided by the substrate concentration and by the protein concentration. Protein concentrations were determined using a value of 18.3 for $A^{1\%}_{1\,cm}$ at 280 nm and M_r 24000 for papaya peptidase A [3] and M_r 26000 for succinyl-papaya peptidase A.

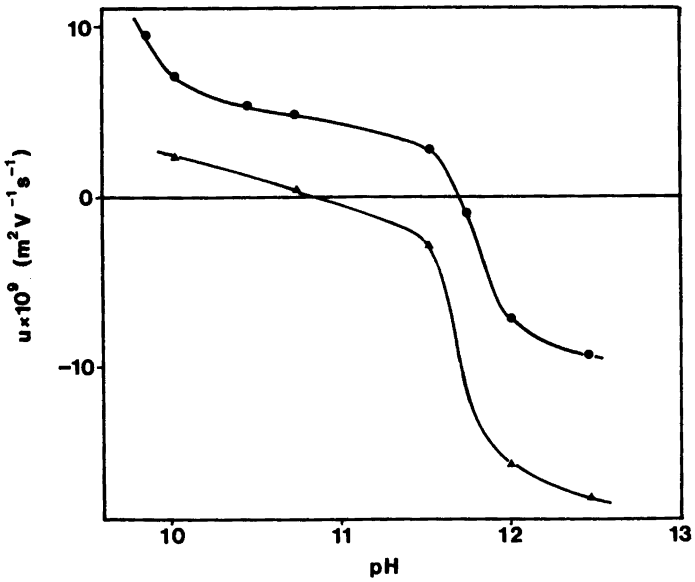

Fig. 1. Electrophoretic mobilities at 25 °C, glycine–NaOH buffers, I=0.14 M *versus* pH for (●) papaya peptidase A and (▲) cytochrome *c*.

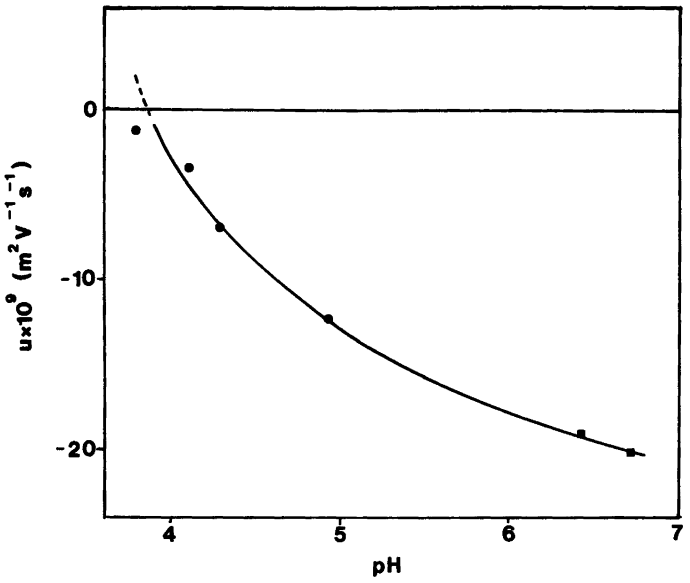

Fig. 2. Electrophoretic mobilities at 25 °C, I=0.1 M *versus* pH for succinyl-papaya peptidase A in (●) sodium acetate–acetic acid buffers and (■) sodium barbiturate–acetic acid buffers.

RESULTS AND DISCUSSION

The exact electrophoretic mobility of papaya peptidase A was determined at eight different pH values in the range 9.8–12.4. For comparison, cytochrome c was included in five of the runs as an example of a well-known basic protein. In Fig. 1, a plot of the electrophoretic mobility at 25 °C, I=0.14 M, against pH is shown for papaya peptidase A and cytochrome c. This reveals isoelectric points of 11.7 and 10.8, respectively. The corresponding value for cytochrome *c* given in the literature [11] is 10.65 determined by free electrophoresis, I=0.2 M. Considering the different ionic strength used here the agreement is very satisfactory.

Since the pI value of 11.7 apparently establishes papaya peptidase A as an enzyme of a hitherto unknown basicity, it was of interest to examine how the protein would respond to the introduction of a massive negative charge, *e.g.* to succinylation. The pH-dependence of the electrophoretic mobility for succinyl-papaya peptidase A is shown in Fig. 2. Compared to the unmodified enzyme, the solubility of succinyl-papaya peptidase A at low pH values is decreased by at least a factor of ten, thus causing difficulties in the determination of electrophoretic mobilities below pH 4.5. Consequently, the pI of succinyl-papaya peptidase A had to be obtained by extrapolation yielding a value of approximately

Table 1. Comparison of papaya peptidase A and succinyl-papaya peptidase A.[a]

	Papaya peptidase A	Succinyl-papaya peptidase A
NH_2 groups/molecule [b]	24.8±1.5	2.5±1.0
O-succinylated groups/molecule [c]	—	3.3±0.2
Specific activity/$M^{-1}s^{-1}$ [d]	310±30	415±90 [e]

[a] Assuming a value of 18.3 for $A^{1\%}_{1cm}$ at 280 nm, M_r 24000 for papaya peptidase *A* [3] and M_r 26000 for succinyl-papaya peptidase A. [b] TNBS-assay.[9] [c] Alkaline hydroxylamine reaction.[10] [d] Towards BAEE at pH 6.0. [e] Based on 4 determinations.

3.8, *i.e.* as a result of succinylation the pI of papaya peptidase A drops nearly 8 pH units.

In Table 1, papaya peptidase A and succinyl-papaya peptidase A are compared with respect to the content of free amino groups, the number of *O*-succinylated hydroxyamino acids and specific activity. It is seen that 3 hydroxyl groups and about 20 amino groups are succinylated under the conditions employed. Furthermore, when taking the *N*-terminal amino group into account, the estimated number of 24.8±1.5 amino groups per molecule of papaya peptidase A provides an independent check of the number of lysine residues listed in the amino acid analyses of Schack [1] and Robinson.[3] These investigators reported 22 and 23 lysine residues, respectively, per molecule of papaya peptidase A in excellent agreement with the result obtained by the TNBS-assay used here. The fact that conversion of 3 neutral hydroxyl groups and 20 positive amino groups into negative groups causes a change in pI as large as 8 pH units seems reasonable, especially when compared with papain, for which Sluyterman and De Graaf [6] found a corresponding change in pI of 5 pH units upon succinylation of 4 neutral hydroxyl groups and 8 positive amino groups.

Still, in accordance with the case of papain,[12] Table 1 shows that the specific activity towards BAEE at pH 6.0 is only slightly affected by succinylation, probably indicating a largely unchanged configuration of the active center residues upon modification. However, it seems unlikely that the protein as a whole should remain unaffected by this rather drastic modification, which around neutral pH constitutes a change of about 43 charges. This presumption was qualitatively confirmed by comparing the charge/mobility ratio as obtained experimentally with that predicted by the theories of Debye, Hückel, Henry and Gorin.[13] Electrophoresis of papaya peptidase A under conditions identical with those of the pH 6.71 run of Fig. 2 (*i.e.* sodium barbiturate–acetic acid buffer, I=0.1 M) yielded a mobility of 14.5×10^{-9} m^2 V^{-1} s^{-1}. For f/f_o=1.14, $\bar{v}$=0.727 cm^3/g and M_r 24 000[1,3] this mobility gives a charge/mobility ratio of 0.74×10^9 V s m^{-2}, whence papaya peptidase A possesses an average net charge of +10.73 at pH 6.71, I=0.1 M.

Since succinylation decreases the charge by 43 units (Table 1) and the mobility of the succinylated enzyme at pH 6.71 is -20.2×10^9 m^2 V^{-1} s^{-1} (*cf.* Fig. 2), the z/u ratio for the modified protein is 1.60×10^9 V s m^{-2}. In view of the fact that the z/u equation [13] has been clearly validated by experimental data for serum albumin,[14,15] it appears reasonable to ascribe the above difference in z/u ratios to a significant conformational change produced by succinylation and to different degrees of electrolyte binding.

In the absence of binding data, a tentative calculation using $z/u=1.6\times10^9$ V s m^{-2}, $\bar{v}$=0.727 cm^3/g and M_r 26 000 yields a frictional coefficient, f/f_o, of 1.35 for the succcinylated enzyme corresponding to an axial ratio of about 6.5 as compared to the f/f_o value of 1.14 for the unmodified enzyme, which corresponds to an axial ratio of about 3.

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