

FIG. 1. (a and b) Difference spectra generated during thermolysin digestion of β_c -EP (1.5 mg/ml) in 0.1 M Tris-HCl, pH 8.2, and 0.1 M Tris-HCl/50 mM CaCl_2 , pH 6.5, respectively, at 25°C and an enzyme/peptide ratio of 1:1000. (c and d) Difference spectra generated during thermolysin digestion of [Met]EK (0.25 mg/ml) in 0.1 M Tris-HCl, pH 8.2, at 25°C at enzyme/peptide ratios of 1:1000 and 1:100, respectively. Reference cells contained the peptide and sample cells contained the peptide and thermolysin. The time (min) at which each spectrum was taken is indicated. ---, Baseline.

tensity of the tyrosyl peak at 286 nm is $\Delta\epsilon = 66 \text{ M}^{-1}\text{cm}^{-1}$. Much weaker blue-shift bands occur between 270 and 250 nm arising from phenylalanine residues. Similar spectral shifts were obtained using natural porcine β -EP (data not shown). Analogous but significantly more intense blue-shift difference spectra are commonly seen during the denaturation of globular proteins and indicate transfer of aromatic side chains from the hydrophobic interior to the external aqueous solvent (23–26).

Two features of the β_c -EP difference spectra at pH 8.2 are due to the presence of the slight amount (2–6%) of ionized tyrosyl residue expected at this pH (27, 28). The absorption bands

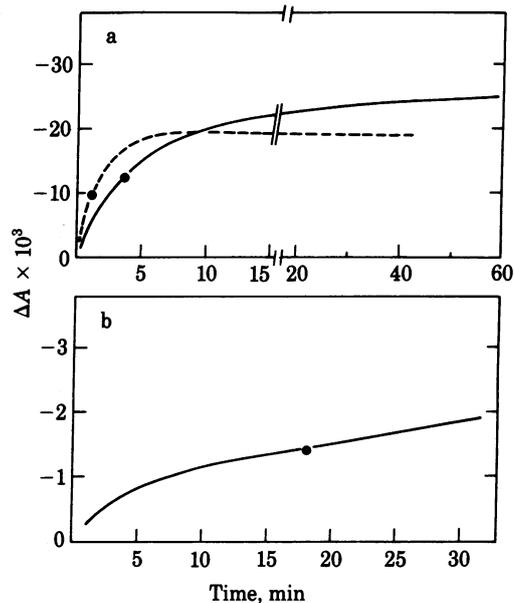


FIG. 2. Time course of the spectral shift at 286 nm produced by digestion of β_c -EP at 1.5 mg/ml (a) and [Met]EK at 0.25 mg/ml (b) in 0.1 M Tris-HCl, pH 8.2 (—), and 0.1 M Tris-HCl/50 mM CaCl_2 , pH 6.5 (---). Enzyme/substrate ratios were 1:1000 and 1:100 for β_c -EP and [Met]EK, respectively, and digestion was at 25°C. After complete digestion of the β_c -EP contained in the sample compartment, the same amount of thermolysin (vol, 1 μl) was added to the reference cell. The change in intensity of the spectral shift at 286 nm was then determined as a function of time to completion of the reaction. The half-lives were: β_c -EP at pH 8.2, 210 sec; β_c -EP at pH 6.5, 65 sec; [Met]EK at pH 8.2, 18 min.

of the ionized phenolic groups, which normally appear at 293–295 nm and 245 nm (25, 29) as opposed to 275 and 230 nm for the un-ionized form, are also blue shifted during the digestion of β_c -EP. This shift produces the negative shoulder above 290 nm and the extra absorbance below 275 nm that prevents the spectrum from returning to the baseline. At pH 6.5 (Fig. 1b), where virtually none of the tyrosyl groups can be ionized, both of these effects disappear, although the effect of digestion on the un-ionized side-chain spectrum is completely retained. Because both the ionized and un-ionized forms of tyrosine are blue shifted, hydrogen bonding of the phenolic OH and charge effects produced by the appearance of new COOH groups can be ruled out as causes or part of the spectral effects.†

The kinetics of the blue shifting at 286 nm is pseudo-first order at both pH values, with half-lives of 3.50 min and 1.08 min at pH 8.2 and pH 6.5, respectively (Fig. 2a). The increase in digestion rate at pH 6.5 is due to the addition of Ca^{2+} ion, which stabilizes the enzyme. At pH 8.2, Ca^{2+} was not added to slow the digestion to allow peptide mapping during the earliest possible part of the reaction.

In striking contrast, digestion of [Met]EK under the same conditions (pH 8.2 and an enzyme/substrate ratio of 1:1000) does not produce any measurable difference spectrum even after 1 hr (Fig. 1c). Increasing the enzyme/substrate ratio to 1:100 slowly produced a very small effect (Figs. 1d and 2b). The final difference spectrum after 80 min is qualitatively similar to the β_c -EP spectra but is more than one order of magnitude

† New COOH groups can produce only red shifts under these conditions (21–24). Extremely weak blue shifts might possibly be produced through an inductive mechanism if new amino groups, partially positive at pH 8.2 and pH 6.5, were held in close proximity to the tyrosine ring (23). This would require maintenance of a tertiary structure even after complete enzymatic digestion.

less intense per mol, $\Delta\epsilon = 3\text{--}6 \text{ M}^{-1}\text{cm}^{-1}$, which is close to the limit of experimental error.

Peptide mapping (Fig. 3b) of the [Met]EK digest showed nearly quantitative cleavage of only one bond—that between glycine-3 and phenylalanine-4—demonstrating that cleavage near the tyrosyl residue cannot by itself produce the relatively intense and rapidly appearing blue-shift spectra seen for β_c -EP. The local environments and conformations around the tyrosyl residues in these two molecules are distinctly different, with [Met]EK showing no convincing evidence of any conformational effects.

To determine what portion of the β_c -EP sequence beyond residue 5 might be responsible for the tertiary structural interaction with the NH_2 -terminal tyrosine, aliquots of the thermolysin digest were removed at various times and submitted to peptide mapping (Fig. 3a). The peptide map obtained after 45–60 sec of digestion, during which time 10–15% of the total blue shift has occurred (Fig. 2a), shows only three detectable spots, one of them being the undigested starting material. The other two fragments ($\approx 10\%$ yield) correspond to residues 1–21 and 22–31 of the β_c -EP sequence (see Fig. 3 and Table 1). After 4 min of digestion (see Fig. 1a for extent of optical transition), peptide maps indicated increasing amounts of β_c -EP-(22–31) (40% of the total), together with new fragments corresponding to residues 26–31, 18–31, and 1–16 (data not shown). However, no peptides corresponding to the truncated EK sequence (fragments 1–3 and 1–4) could be detected.

A peptide map obtained after completion of the optical transition (1 hr; see Fig. 2a) is also shown in Fig. 3. A total of 11 peptides could be resolved; their sequences (deduced from amino acid composition) and yields are given in Table 1; COOH-terminal fragments account for 62% of the recovered peptides

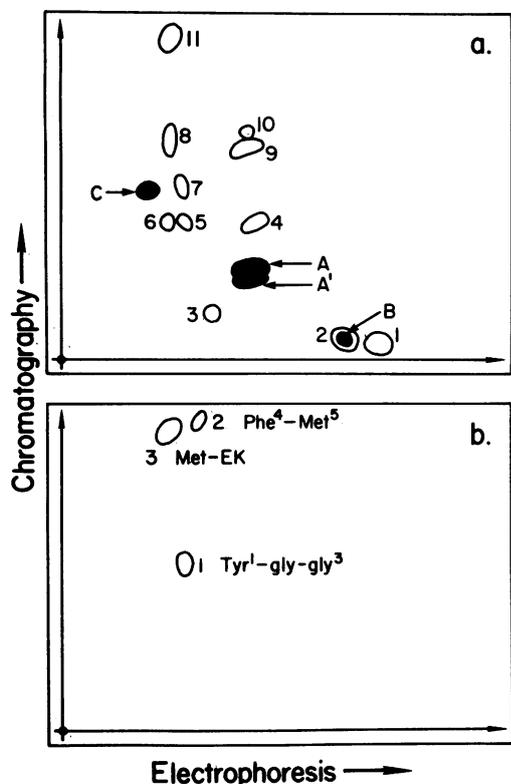


FIG. 3. (a) Two-dimensional paper chromatography/electrophoresis patterns of β_c -EP (1.5 mg) (a) and [Met]EK (1 mg) (b) after digestion with thermolysin at enzyme/substrate ratios of 1:1000 and 1:100, respectively, in 0.1 M Tris-HCl, pH 8.2, at 25°C. β_c -EP was digested for 45–60 sec (●) or 1 hr (○) and [Met]EK was digested for 80 min.

Table 1. Sequences and yields of peptides from thermolysin digestion of β_c -EP

Peptide*	Sequence	Recovered amount, μg	Yield, [†] %
60 min of digestion			
1	Ala ²⁶ -Gln ³¹	230.0	27.5
2	Ile ²² -Gln ³¹	81.0	9.7
3	Met ⁵ -Pro ¹³	57.5	6.7
4	Phe ¹⁸ -Ala ²¹	55.0	6.6
5	Met ⁵ -Leu ¹⁴	42.5	5.0
6	Met ⁵ -Thr ¹⁶	50.5	6.0
7	Tyr ¹ -Gly ³	12.6	1.5
8	Met ⁵ -Leu ¹⁷	95.0	11.0
9	Ile ²³ -Asn ²⁵	57.0	7.0
10	Leu ¹⁷ -Ala ²¹	91.0	11.0
11	Tyr ¹ -Phe ⁴	66.0	8.0
1 min of digestion			
A	β_c -EP	570.0	87.5
A'	β_c -EP		
B	Ile ²² -Gln ³¹	37.5	5.7
C	Tyr ¹ -Ala ²¹	44.0	6.8

β_c -EP (1 mg) was digested with thermolysin at an enzyme/substrate ratio of 1:1000.

* See Fig. 3.

[†] Calculated on a weight basis.

with a single fragment, β_c -EP-(26–31), yielding 27% of the total. In contrast, fragments corresponding to the EK sequence still appear in only 10% yield. These data are consistent with the relative resistance of β_c -EP-(1–5) to thermolysin attack and the slow kinetics of the miniscule blue-shift spectra appearing during digestion of [Met]EK (Fig. 1d). Moreover, they further emphasize the fact that it is cleavage within the COOH-terminal segment of β_c -EP, with particular emphasis on the alanine-21 to isoleucine-22 peptide bond, that is probably responsible for freeing the NH_2 -terminal tyrosine from its interaction with other residue(s). It is also possible that phenylalanine-4 is involved in this interaction; charge effects from new NH_2 groups produced by cleavage can account for only a small portion of the phenylalanine blue-shift spectra in β_c -EP. However, such a charge effect could explain essentially all the phenylalanyl blue-shift in [Met]EK.

The exact mechanism producing the rapid blue shift and the precise assignment of the residue(s) interacting with tyrosine-1 will require further investigation. The significance of a tertiary structural feature linking the NH_2 - and COOH-terminal regions of β -EP in aqueous solution and its relationship to the molecular structure proposed to exist for β -EP in nonpolar solvents (which are thought to mimic the environment of the receptor) remains to be shown.

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