A SOLUBLE, STABLE POLYELECTROLYTE DERIVATIVE OF TRYPSIN

Thomas L. Westman

Research Center, Monsanto Company
St. Louis, Missouri 63166

Received February 10, 1969

A soluble ethylene maleic anhydride copolymer derivative of trypsin has been prepared. This derivative exhibits a shift in the pH optimum toward ca. 9-10 and it is stable in solution for extended periods of time. Digestion of oxidized ribonuclease indicates a change in specificity or reactivity versus native trypsin.

Considerable interest has evolved concerning stabilized and/or chemically modified enzymes. Such modifications can be regarded as falling into two categories: (1) simple chemical modification such as succinylation, nitration, iodination and carbamylation (Gonnaris and Ottesen, 1965), and (2) modification by attachment of enzymes to polymeric materials to yield insolubilized adducts (Tosa and Chibata, 1967). It is noteworthy, however, that apparently all of the well-defined enzyme/polymer systems reported to date are water insoluble. This paper describes an example of a soluble enzyme/polymer derivative which possesses both increased stability and modified reactivity.

Trypsin and ethylene maleic anhydride copolymer (EMA, m. w. 30,000) were allowed to react in 0.2 M phosphate buffer, pH 7.5, at 4° C in a manner analogous to that of the procedure of Levin, et al. (1964) except that crosslinking agents were intentionally omitted. Insoluble (crosslinked) materials were

separated from the supernatant by centrifugation and the crude, soluble trypsin/EMA adduct (SEMAT) was isolated from the supernatant by dialysis and lyophilization. In one preparation a crude SEMAT sample contained 5.34% N (dry weight) and an activity of 3.14×10^{-2} units/sec/mg* at pH 9.5 (vide infra).

Although a number of proteins interact ionically with hydrolyzed ethylene maleic anhydride copolymer (HEMA) it was found that careful and repetitive Sephadex G-100 chromatography at high ionic strengths apparently provides major separation of unbound and occluded protein from the SEMAT adduct. G-100 chromatography of the crude SEMAT product (above) resulted in elution of the SEMAT in the void volumes, as expected, whereas trypsin was eluted in volumes comparable to that of trypsin alone. Substantial separation of unbound trypsin from the SEMAT was suggested by the fact that G-100 chromatography of physical mixtures of trypsin and HEMA gave good separation as shown by the low optical density (at 280 nm), low BAEE activity and marginal nitrogen content (less than 0.5%) of void volume fractions. Interestingly, HEMA (m.w. ca. 30,000) was found to be eluted mainly in the void volumes of G-100 columns, presumably due to a non-globular structure.

Sephadex (TM) G-100 chromatography (0.2 M phosphate, pH 7.5) of the above crude SEMAT gave a purified SEMAT product containing 3.50% N (dry weight) and a BAEE activity of 3.95×10^{-2} units/sec/mg at pH 9.5.

Further evidence that the chromatographed SEMAT is largely

---

* Assays were determined by following the rate of hydrolysis of α-N-benzoylarginine ethyl ester (BAEE) by change in optical absorbance at 255 nm. Activity, for convenience, was based upon arbitrary units of change of absorbance/sec. Trypsin under comparable conditions at pH 7.5 had an activity of 1.6×10^{-1} units/sec/mg.

free of unbound trypsin is shown by the fact that disc gel electrophoresis of mixtures of HEMA and trypsin gave a stainable band corresponding to that of native trypsin. SEMAT, however, gave no stainable migration into the lower gel (electrophoresis run toward both electrodes) but it was stainable when mixed with the gel. In addition, nitrous acid deamination, acid hydrolysis and amino acid analysis of SEMAT preparations indicated that a number of lysines (via ϵ -NH_2 groups) had been blocked.

Solutions (0.1 M KCl) of trypsin and SEMAT were allowed to stand at room temperature and BAEE activity was measured as a function of time.* Substantial activity towards BAEE was maintained by the SEMAT solution; with ca. 65% of the initial activity remaining after 17 days in solution. Trypsin, on the other hand, underwent the expected autolysis; losing most of its activity within 2-3 days. In another stability study, an unpurified SEMAT preparation was found to maintain at least 45% of its original activity after 30 days in solution. A solution of HEMA and trypsin (3:1 w/w) displayed only marginal enhancement of stability. HEMA catalyzed hydrolysis of BAEE appeared insignificant under the assay conditions.

The pH profile of SEMAT (BAEE substrate) was found to mimic that of IMET (Levin, et al., 1964), with the pH optimum shifted toward higher pH and a tailing of activity into higher pH ranges. (Figure 1). The pH shift is apparently due to the carboxyl environment near the trypsin moiety or an influence on the binding of the substrate. Some differences in the pH profile were noted for different buffers and of different ionic strengths.

In order to more realistically assess possible changes in

---

* Care must be employed to avoid microbial contamination which may lead to erroneus results.

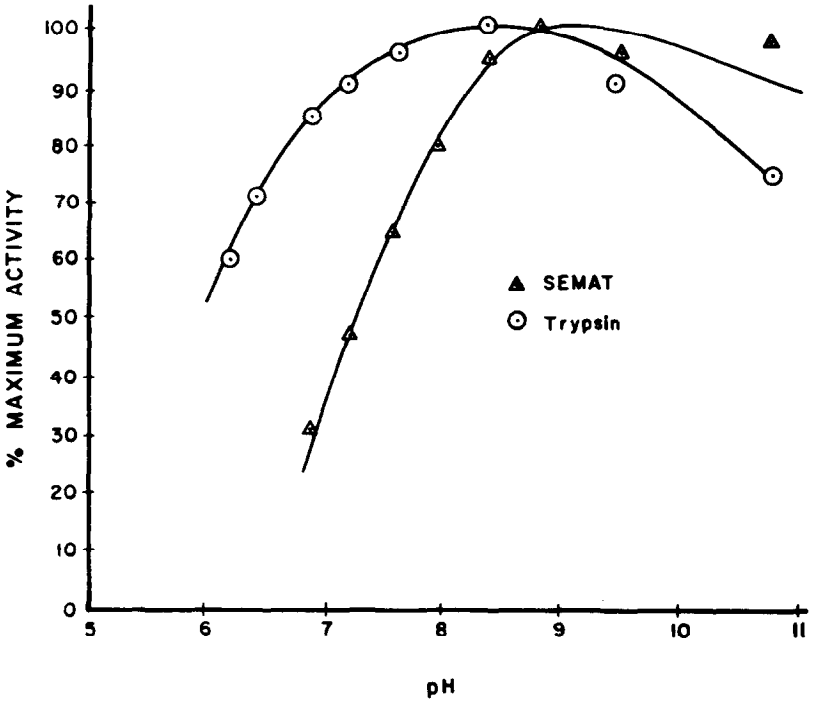

Figure 1. pH Activity Profile for SEMAT and Trypsin. Baee Substrate. Buffers (I=0.05), tris below pH 9; carbonate/bicarbonate above pH 9.

specificity of SEMAT, the digestion of oxidized ribonuclease (RIBOX) was investigated preliminarily. Performic acid oxidized bovine ribonuclease (Hirs, 1956) was digested for 24 and 48 hours with trypsin (50:1 w/w) at pH 7.6 (0.01 M ammonium bicarbonate) and with SEMAT (BAEE activity corresponding to that of the amount of trypsin employed) at pH 9.5 (0.01 M ammonium bicarbonate/ hydroxide). The lyophilized digests were subjected to high voltage electrophoresis/paper chromatography. The trypsin digest of RIBOX gave the expected ca. 13 spots whereas the SEMAT digest gave significantly fewer (6-10) spots upon ninhydrin development. End group analysis (Morse & Horecker, 1966) also confirmed that fewer bonds were cleaved by the SEMAT. Digestion of RIBOX with a HEMA/ trypsin mixture (3:1 w/w) gave results comparable to those obtained with trypsin.

This apparent change in substrate specificity and/or reactivity of SEMAT is similar to results of Ong, Tsang, and Perlmann (1966) who observed that IMET cleaved fewer bonds in pepsinogen derivatives than did trypsin. Whether these limited digestions with SEMAT and IMET are due to changes in substrate specificity or reactivity or other factors has not been determined.

In order to clarify these and other points, attempts are continuing to identify the SEMAT digest of RIBOX. A kinetic examination of the SEMAT system is also concerned with titration of active sites, inhibition studies and peptide substrate reactions.

These results suggest that soluble enzyme/polyelectrolyte derivatives may be of interest in protein structural studies and for investigations on effects of modification of enzyme structures. Further results on SEMAT and other systems will be reported in later publications.

ACKNOWLEDGEMENTS

Excellent technical assistance was provided by N. M. Havner, R. A. Strickland (end group analysis) and by C. Lewis (electrophoresis/paper chromatography).

REFERENCES

Gonnaris, A., and Ottesen, M., Compt. Rend. Trav. Lab. Carlsberg, 35, 37, (1965) and references cited.

Hirs, C. H. W., J. Biol. Chem., 219, 611 (1966).

Levin, Y., Pecht, M., Goldstein, L., and Katchalski, E., Biochemistry, 3, 1905, 1913 (1964).

Morse, D., and Horecker, B. L., Anal. Biochem., 14, 429 (1966).

Ong, E. B., Tsang, Y., and Perlmann, G. E., J. Biol. Chem., 241, 5661 (1966).

Tosa, T., and Chibata, I., Hakko Kyokaishi, 25, 49 (1967).