A SPECTROPHOTOMETRIC ASSAY FOR NEUTRAL PROTEASE

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The specificity of B. subtilis neutral protease has been investigated in some detail (Feder, 1967; Feder and Lewis, 1967; Morihara and Oka, 1968; Morihara, Tsuzuki and Oka, 1968). These studies have indicated an endopeptidase activity specific for peptide bonds in which leucine and phenylalanine contribute the amino group. No esterolytic activity has been observed for this enzyme with a number of esters.

The enzyme activity has been assayed using protein substrates and measuring trichloroacetic acid soluble material obtained upon digestion (McConn, Tsuru and Yasunobu, 1964; Anson, 1938). Recently some simple dipeptides have been reported useful for kinetic assays for this enzyme (Feder, 1967; McConn, Tsuru and Yasunobu, 1967). Using Z-Thr-Leu-NH_2 under pseudo-first order conditions the reactions have been monitored using a recording pH stat. The hydrolysis of a dipeptide, however, does not lend itself to spectrophotometric techniques which diminish many of the hazards for routine evaluation inherent in other methods. Since no esterolytic activity has been observed which lends itself to spectrophotometry some thought has been put to the construction of specific dipeptide substrates that undergo spectral perturbation when the dipeptide bond is hydrolyzed. Bernhard and coworkers (1965, 1967) have described the synthesis and use of β-arylacryloyl derivatives particularly for the spectrophotometric identification of

chymotrypsin and subtilisin acyl enzyme intermediates. McClure and Neurath (1966) reported the use of N-trans-3(-2-furylacryloyl)-L-phenylalanine and O-trans-3-(2-furylacryloyl)-DL-β-phenyl-lactate as chromophoric amide and ester substrates for carboxy-peptidase A. The use of a furylacryloyl blocking groups for dipeptides permits the dipeptide hydrolysis to be readily monitored spectrophotometrically. A simple spectrophotometric assay for neutral protease using the dipeptide substrate 3-(2-furylacryloyl)-glycyl-L-leucine amide is here described.

Experimental

Materials and Methods

3-(2-furylacryloyl)-glycyl-L-leucine amide (FAGLA) was prepared by Cyclo Chemical Corporation. Stock solutions of this dipeptide were prepared in pH 7.22 phosphate buffer (μ=0.1) at a concentration of 2.49×10^{-3} M (76.6 mg/100 ml). Lower concentrations of substrate have been used with the same facility and no product inhibition has been observed. Although phosphate buffer was used in this work, there is some inhibition with this buffer. Tris buffers do not show this inhibition and is prescribed in preference to phosphate. Only reagent grade salts and deionized water was used throughout these studies.

Crystalline subtilisin was purchased from Nutritional Biochemical Corporation. Neutral protease from *Bacillus subtilis* fermentation beers at various stages of purification was used for these studies. All neutral protease preparations were treated with 10^{-3} M diisopropylfluorophosphate until no detectable enzyme catalyzed hydrolysis of Z-gly-ONP was observed. Protease determinations using casein substrate were carried out as described by Anson (1938).

The hydrolysis of FAGLA was followed spectrophotometrically

at 25° using a Cary 14 P.M. recording spectrophotometer and also a Beckman D. U. Spectrophotometer. A decrease in absorbance at 345 mμ was observed as the substrate was hydrolyzed. The substrate was stable in buffer solutions at neutral to slightly acid pH and can be stored as such. Since the substrate has a considerable absorption at this wave length ($\epsilon_{345\ m\mu}$ = 766) a screen ($A_{345\ m\mu}$ = 1.204) was used to blank out some of the absorbance when carrying out reactions in the Cary 14 Spectrophotometer. For the Beckman D.U. a blank can be prepared by diluting the substrate with water (3:1) (FAGLA/H_2O).

A $\Delta\epsilon_{345}$ of 317 was obtained for the enzyme catalyzed hydrolysis of this substrate. In all studies the reaction vessel contained 3.0 ml of buffer-substrate solution and 0.1 ml of enzyme.

Results

Three methods have been used to assay for the neutral protease activity using FAGLA. The first involves the calculation of a pseudo-first order rate constant from the reaction data and the other two represent initial rate methods. When $S \ll Km$ the rate equation for an enzyme catablyzed reaction becomes $v = \frac{kcat\ (EO)\ (S)}{Km}$, yielding a pseudo-first order rate constant k equal to $\frac{kcat}{Km}(E_o)$. A plot of k versus (E_o) should be linear and the slope $\frac{kcat}{Km}$ is characteristic for this enzyme under these conditions and can be used to calculate the enzyme concentration from other values of k. The Km for this reaction was found to be about 3.00×10^{-2} M. The reactions were carried out at a substrate concentration of 2.49×10^{-3} M and good pseudo-first order kinetics were observed. Over an initial substrate concentration range of 3.82×10^{-3} M to 3.80×10^{-4} M the pseudo-first order rate constants agreed within three per cent for the same enzyme concentration. The data was evaluated using infinity plots and

the method of Kezdy, Jaz and Bruylants (1958) and the plots were linear for more than ninety-five per cent of the reaction with excellent agreement between methods. Figure 1 shows a plot of k versus the enzyme concentration expressed as ml of stock enzyme per ml of solution. A linear relationship was obtained over a forty fold range of enzyme concentration.

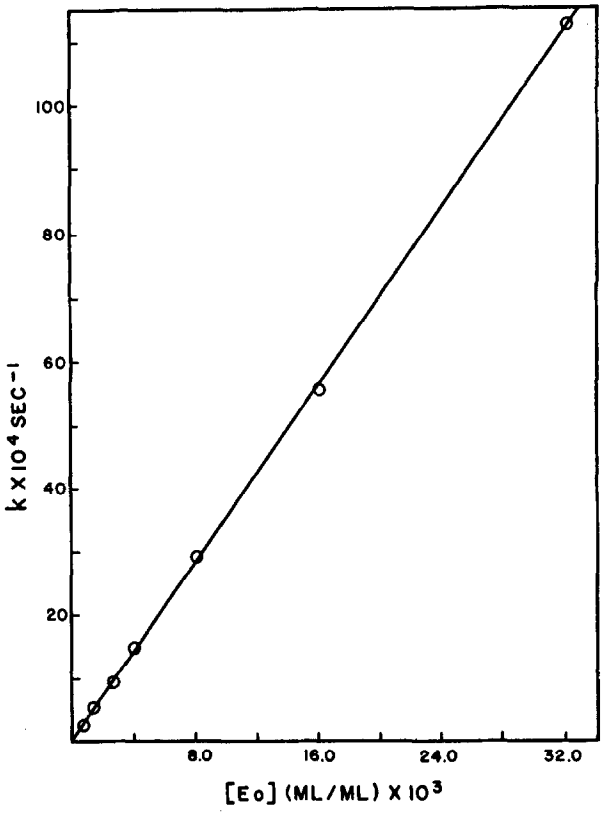

Figure 1. The effect of neutral protease concentration on the pseudo-first order rate constant for the enzyme catalyzed hydrolysis of FAGLA (details in text).

The relationship between enzyme concentration and the initial rate of enzyme catalyzed hydrolysis of FAGLA is shown in Figure 2. These initial rates were obtained from the rate data above, but limiting the measurement to less than 10 per cent of

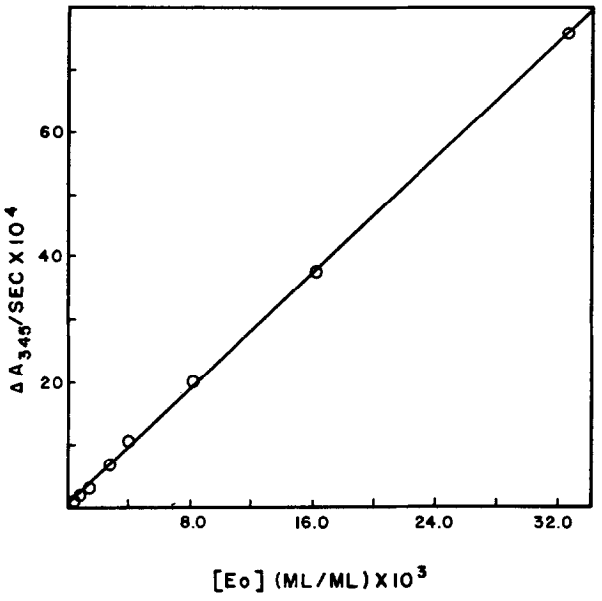

Figure 2. The effect of neutral protease concentration on the initial rate of enzyme catalyzed hydrolysis of FAGLA (details in text).

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total hydrolysis. One can calculate the pseudo-first order rate constants from these initial rate values and the initial substrate concentration. An average value of k/E of 29.97×10^{-2} sec^{-1} $(ml/ml)^{-1}$ was obtained from the initial rate approximation as compared to 35.52×10^{-2} sec^{-1} $(ml/ml)^{-1}$ obtained from actual first order plots. On the other hand this gives good linearity with respect to the enzyme concentration which is an important criterion for its use for assay. One can express the k/E using units of protease by casein assay. An average value of 5.361×10^{-6} sec^{-1} $(units/ml)^{-1}$ was obtained over a 240 fold range of dilution with values of 5.378×10^{-6}, 5.280×10^{-6}, 5.60×10^{-6} and 5.186×10^{-6} sec^{-1} $(units/ml)^{-1}$ for dilutions of 1, 24, 160 and 240 fold respectively.

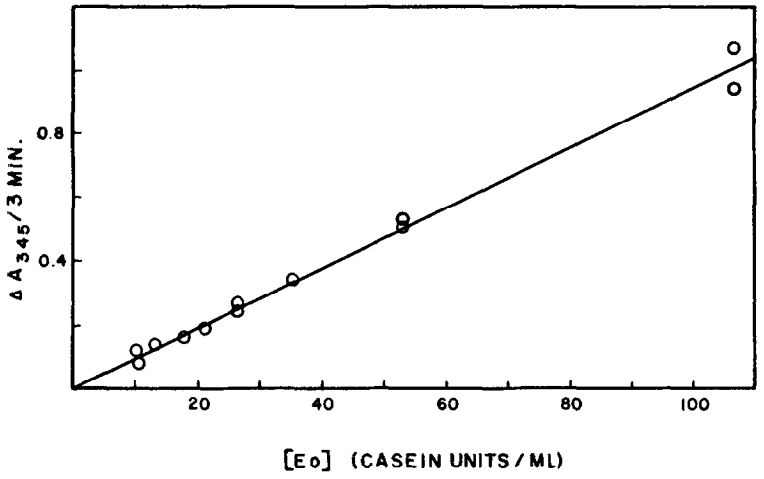

Figure 3. The relationship between enzyme concentration in casein protease units and ΔA345 mμ/3 min with FAGLA (details in text).

Finally, a simple two point initial rate assay for the Beckman D.U. Spectrophotometer was devised using the same conditions as described for the previous methods. To 3.0 mls of substrate-buffer was added 0.1 ml of enzyme solution and the absorbance at 345 mμ was read 30 seconds after mixing. After three minutes the absorbance was read again. The ΔA345/3 min was plotted versus the enzyme concentration expressed as casein protease units per ml of solution. Figure 3 shows these results. This method represents the least refined and analytically perfect method, but one still obtains good linearity between enzyme concentration and activity. One can measure as little as 10 protease (casein) units per ml in the reaction solution.

Crystalline subtilisin catalyzed the hydrolysis of FAGLA at 1/890 the rate of an equivalent weight of neutral protease. Both trypsin and chymotrypsin were inactive toward catalyzing the hydrolysis of FAGLA.

In summary furylacryloyl-glycyl-L-leucine amide is a substrate of choice specific for the neutral protease. It incorporates ease of measuring the reaction, together with a specificity function which permits assay of the neutral protease in the presence of other proteases. Furylacryloyl blocking groups have been used to follow ester and amide hydrolysis and should prove most useful in preparing peptide substrates for enzyme hydrolysis which can easily be monitored by spectrophotometry.

REFERENCES

Anson, M. L., J. Gen. Physiol., 22, 79 (1938).
Bernhard, S. A., Lau, S. J. and Noller, H. F., Biochemistry, 4, 1108 (1965).
Charner, E. and Bernhard, S. A., J. Am. Chem. Soc., 89, 2726 (1967).
Feder, J., Biochemistry, 6, 2088 (1967).
Feder, J. and Lewis, C. Jr., Biochem. Biophys. Res. Commun., 28, 318 (1967).
Kezdy, F. J., Jaz, J. and Bruylants, A., Bull. Soc. Chim. Belges, 67, 687 (1958).
McClure, W. O. and Neurath, H., Biochemistry, 5, 1425 (1966).
McConn, J. D., Tsuru, D. and Yasunobu, K. T., J. Biol. Chem., 239, 3706 (1964).
McConn, J. D., Tsuru, D. and Yasunobu, K. T., Arch. Biochem. Biophys., 120, 479 (1967).
Morihara, K. and Oka, T., Biochem. Biophys. Res. Commun., 30, 625 (1968).
Morihara, K., Tsuzuki, H. and Oka, T., Arch. Biochem. Biophys., 123, 572 (1968).
Noller, H. F. and Bernhard, S. A., Biochemistry, 4, 1118 (1965).