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Effect of Proteolytic Treatment on Dyeing of Casein with Synthetic Dyes

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The activity of proteolytic enzymes is commonly measured using casein as a substrate. Modified caseinolysis assay was developed with synthetic dyes such as procion red, procion yellow and procion blue for subtilisin carlsberg, protease type XVI, trypsin, chymotrypsin, respectively. Optimum pH, incubation time were determined. K_M, V_{max} and k_{cat}/K_M values were also determined for these enzymes. The results indicate that the effect of all the tested enzymes on the synthetic dye-casein complexes; the most appropriate complex was found to be procion yellow.

Key Words: Proteolytic activity, Dyed-casein, Synthetic dyes, Soluble substrates.

INTRODUCTION

Proteolytic enzymes from the genus *Bacillus* are so far the most important group of commercial enzymes with applications in the food, pharmaceutical and detergent industries[1, 2]. Some of the earliest methods for measuring protease activity analyzed the cleavage of proteins. Soluble or insoluble substrates were used for this study[3, 7]. Spectrophotometric assays are the most popular ones for the determination of proteolytic activity.

Both low-molecular weight synthetic chromogenic peptides and dye-stained soluble and insoluble proteins are usually used as substrates[8]. Detection of protease activity is important for biochemical applications such as quantifying protease activity in cell or tissue extracts, during enzyme purification, in protease inhibition studies and for quality control testing. Protease assays have frequently employed proteins such as haemoglobin, fluorescent dye-labelled proteins such as fluorescein thiocarbamoyl casein (FTC-casein), or chromophoric dye-labelled proteins such as casein, collagen and gelatin to detect protease activity[9–11]. The amount of soluble dye-labelled peptide fragments or dye released from the insoluble substrate by the action of protease can be simply quantified by spectrophotometry[12].

In this study, we have reported the development of a modified caseinolysis assay that allows rapid and reliable measurement of proteolytic activity by using synthetic dyes.

EXPERIMENTAL

Dyeing Casein with Synthetic Dyes

Substrates were prepared as follows: 0.02 g casein was dissolved in 10 mL of 0.1 M carbonate/bicarbonate buffer (pH: 9.0) and then 0.02 g/10 mL dye solution (procion red, procion yellow and procion blue) was added and kept at room temperature for 30 min. Dyed-casein was precipitated with 0.5 mL concentrated acetic acid. After centrifugation at 4500 rpm the precipitate was washed with 10 mL of water threefold, to remove any unbound dye, centrifuged at 4500 rpm again and dried.

Enzyme assay: The dyed casein solution (1 mL of 0.2% dyed casein) and 50 μL of enzyme solution and 2 mL of buffer (pH: 0.1 M tris-HCl, pH: 8) was added into test tubes. The reaction mixture was incubated at 37°C for 15 min. The reaction was stopped by adding 1 mL of 0.6 M trichloroaceticacid (TCA) and then incubated at 4°C for an additional 10 min. The reaction mixture filtrate was measured at selected wavelength against a blank (50 μL of buffer was added to the substrate suspension instead of protease solution).

Determination of optimum pH: Proteolytic activity was measured at different pH's (pH 6, 6.5, 7, 7.5, 8: 0.1 M phosphate buffer; pH 8.5: 0.1 M tris-HCl buffer; pH 9, 9.5, 10, 10.5 and 11: 0.1 M carbonate/bicarbonate buffer). Enzyme activity was measured at standard assay condition.

Determination of enzyme concentration and incubation time: At the constant dyed-casein concentrations and different times (5, 10, 15, 20, 30 and 40 min), enzyme (as 25, 50, 75, 150, 250 and 500 μL, 6.693 U/mL) solutions were incubated and then proteolytic activity was measured at the standard assay condition.

RESULTS AND DISCUSSION

These new substrates were hydrolyzed by various proteases, *e.g.*, by subtilisin carlsberg (E.C. 232-752-2, catalog number P-5380), trypsin (E.C. 3.4.21.4), chymotrypsin (E.C. 3.4.21.1), protease from *Bacillus* sp. (type XVI, No. P-8775). During the substrate hydrolysis, dyed peptide fragments are released into the surrounding medium, which grows coloured.

During the purification process of proteolytic enzymes from a tissue, organelle or cell, the amount and catalytic activity of the enzyme to be purified should be monitored at each stage of purification. This process is done frequently; so the method to be followed should be economic, easily applicable and cause no environmental pollution.

To evolve specific substrates for at least one of frequently used proteolytic enzymes: subtilisin carlsberg, trypsin, chymotrypsin and protease type VI, we have dyed casein with procion red, procion yellow and procion blue.

The catalytic activity of subtilisin carlsberg, trypsin, chymotrypsin and protease type XVI on casein staining-material complexes prepared as soluble substrates

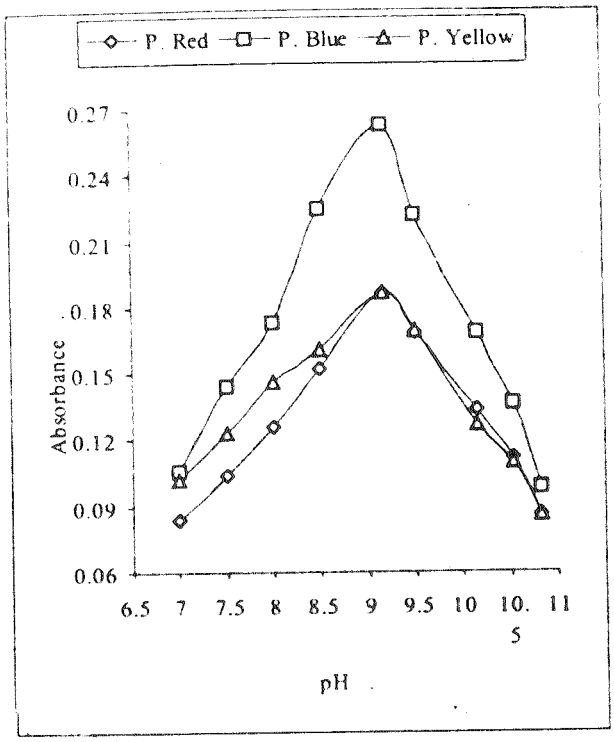

Fig. 1. Effect of pH on subtilisin carlsberg activity

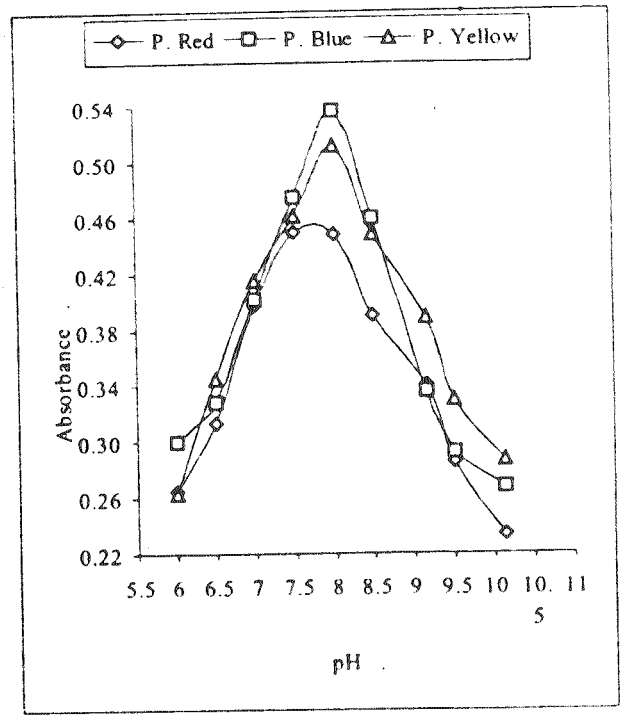

Fig. 2. Effect of pH on protease Type XVI activity

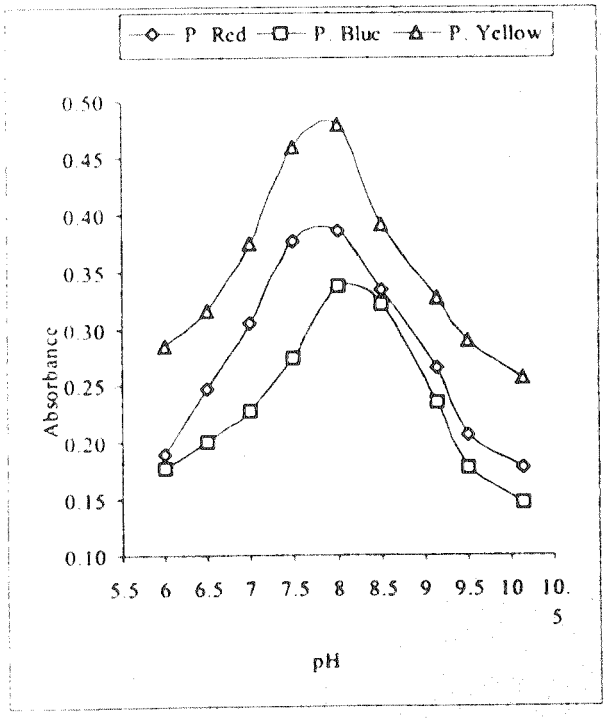

Fig. 3. Effect of pH on trypsin activity

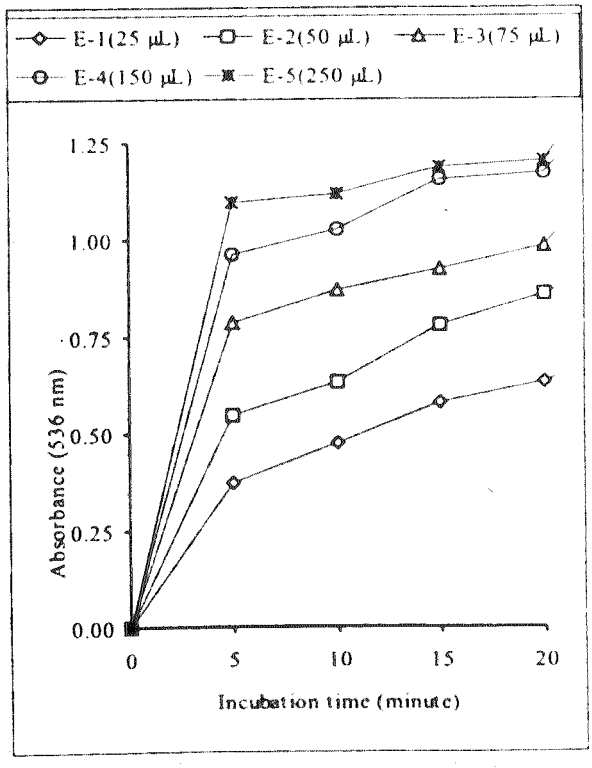

Fig. 4. Effect of pH on chymotrpsin activity

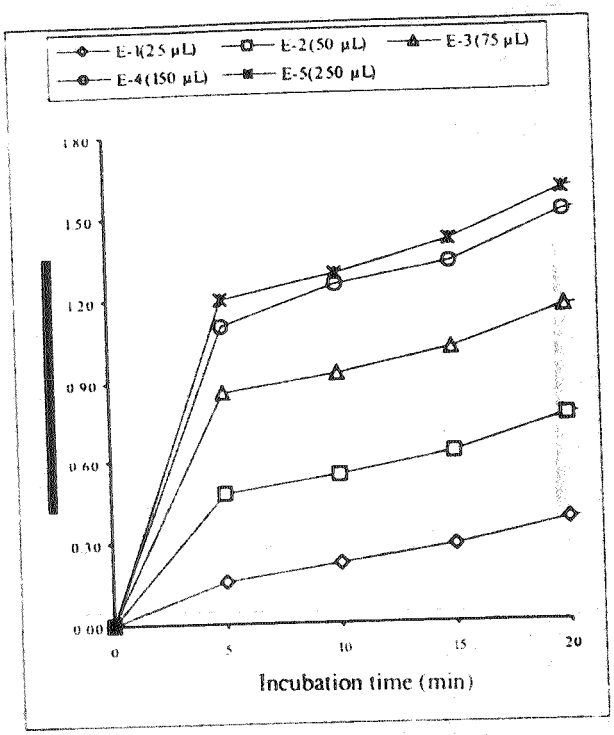

Fig. 5. Appropriate subtilisin carlsberg concentration with procion red-casein complex

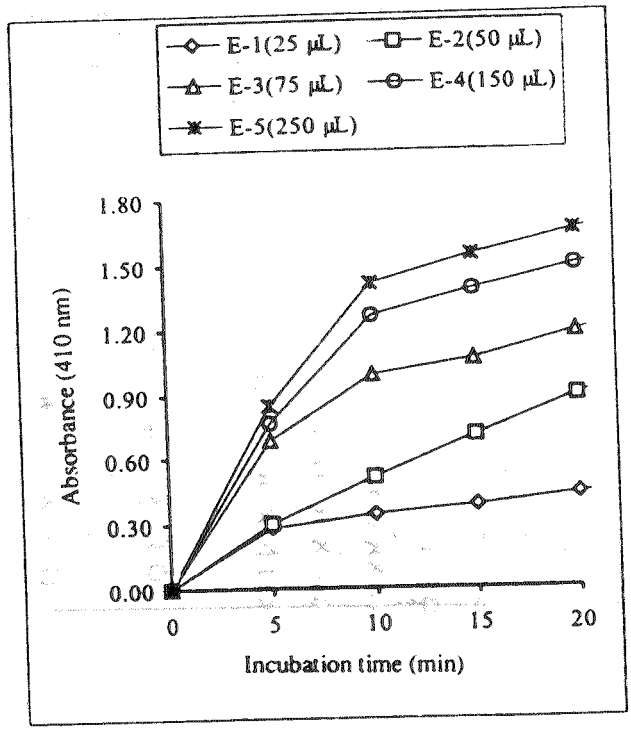

Fig. 6. Appropriate subtilisin carlsberg concentration with procion blue-casein complex

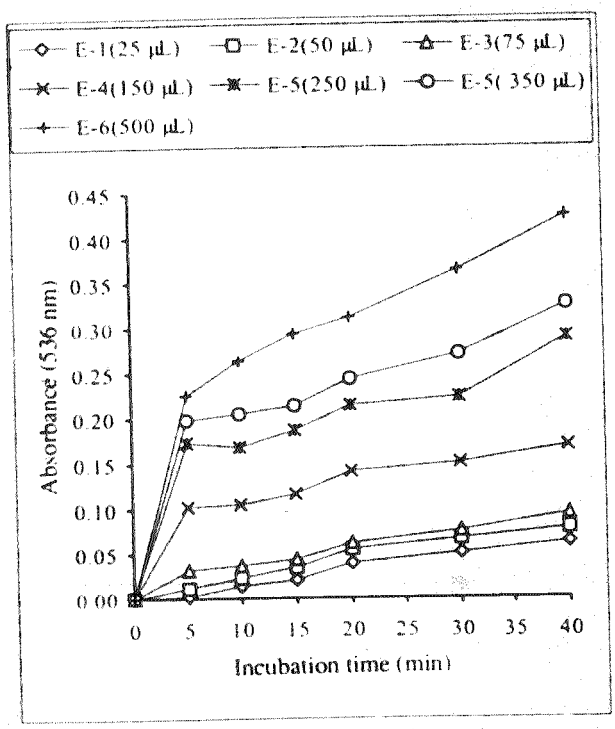

Fig. 7. Appropriate subtilisin carlsberg concentration with procion yellow-casein complex

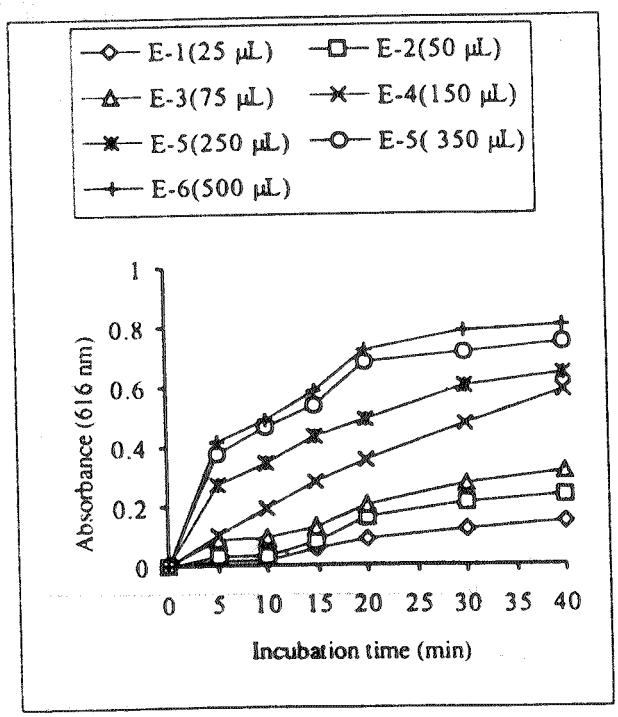

Fig. 8. Appropriate protease type XVI concentration with procion red-casein complex

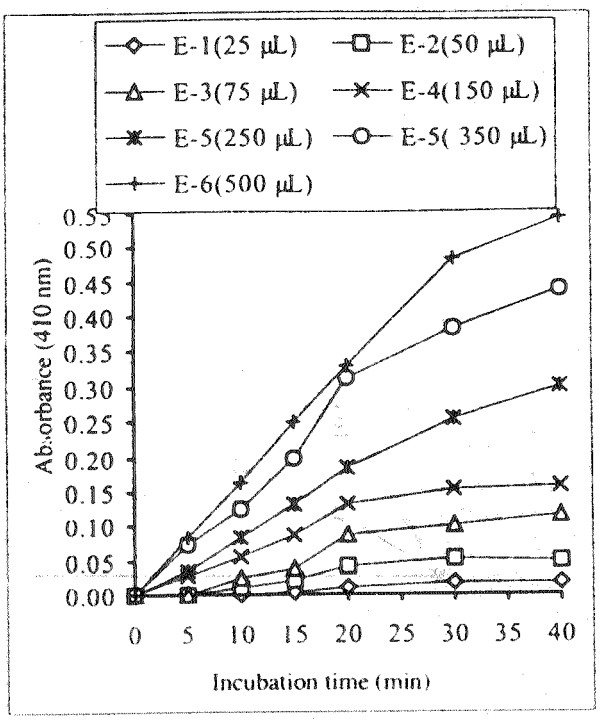

Fig. 9. Appropriate protease type XVI concentration with procion blue-casein complex

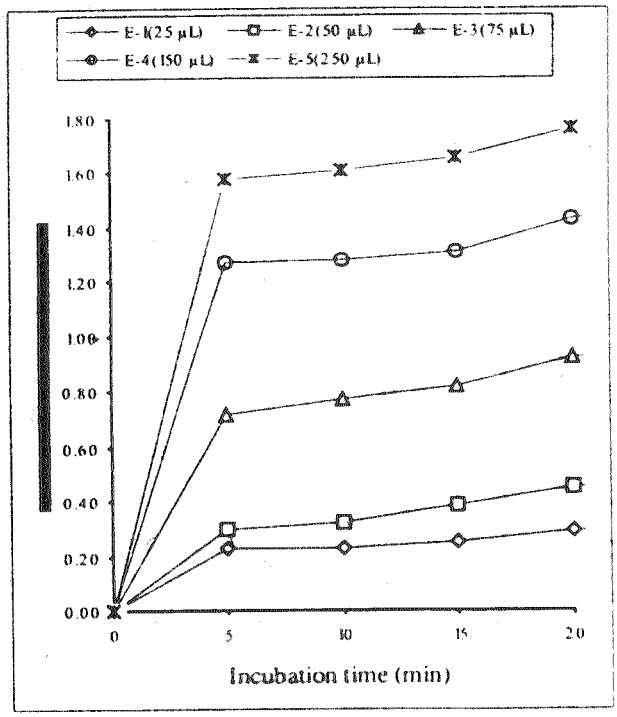

Fig. 10. Appropriate protease type XVI concentration with procion yellow-casein complex

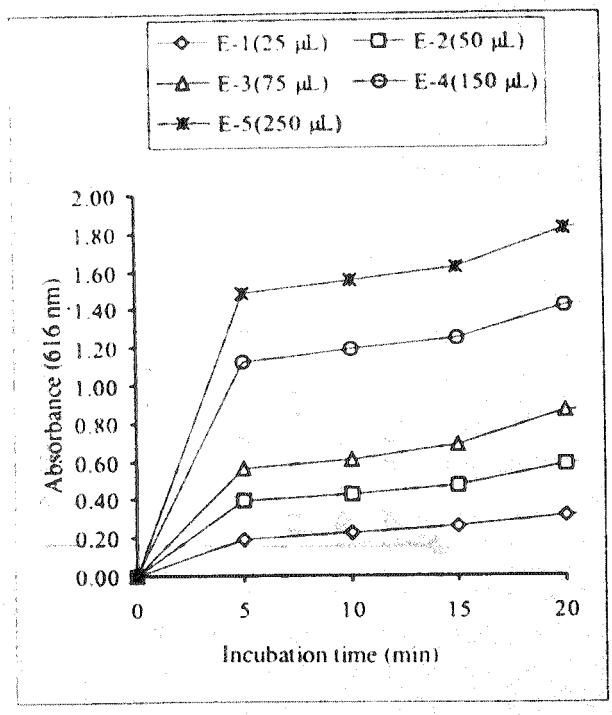

Fig. 11. Appropriate trypsin concentration with procion red-casein complex

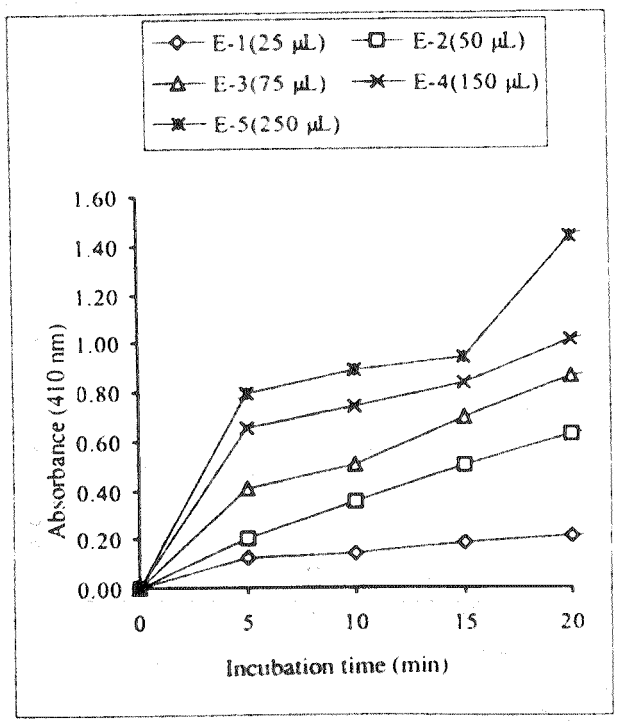

Fig. 12. Appropriate trypsin concentration with procion blue-casein complex

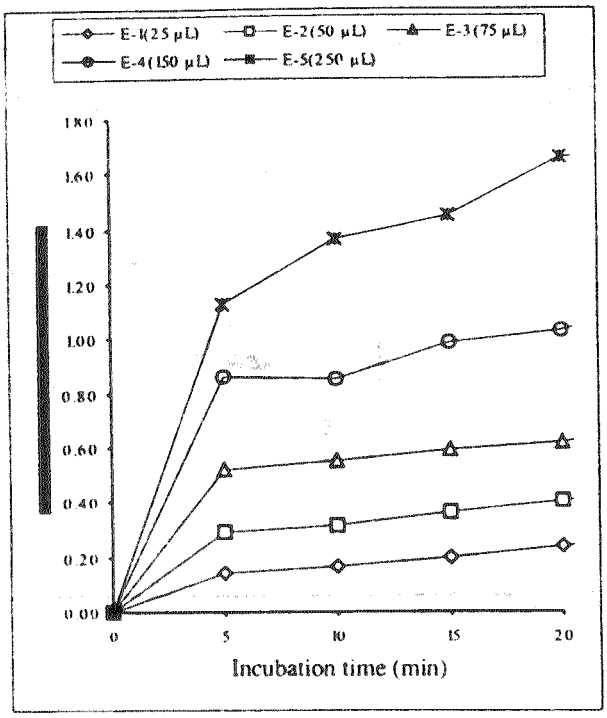

Fig. 13. Appropriate trypsin concentration with procion yellow-casein complex

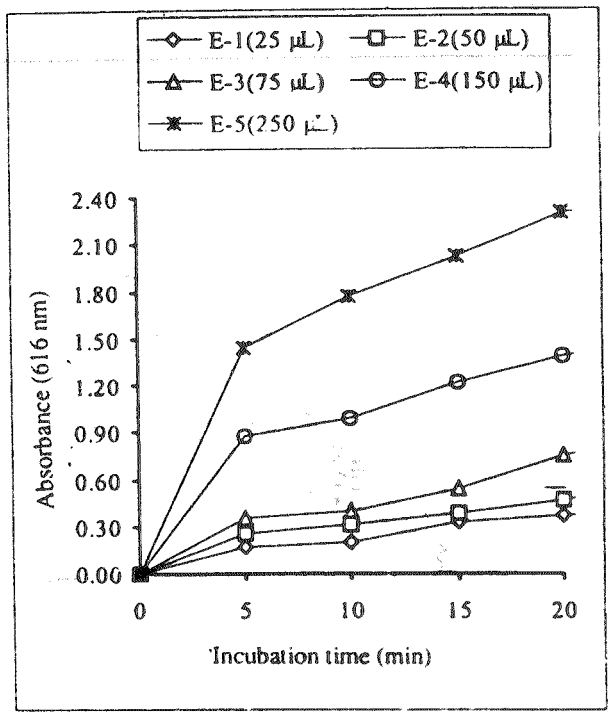

Fig. 14. Appropriate chymotrypsin concentration with procion red-casein complex

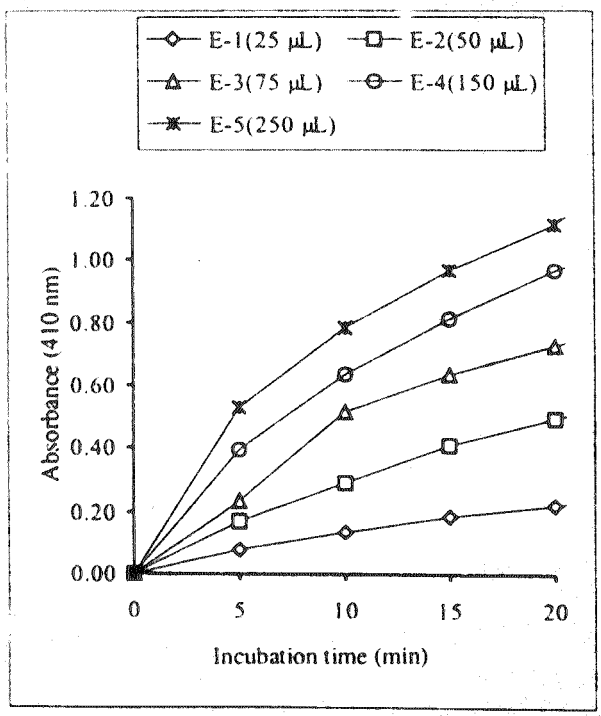

Fig. 15. Appropriate chymotrypsin concentration with procion blue-casein complex

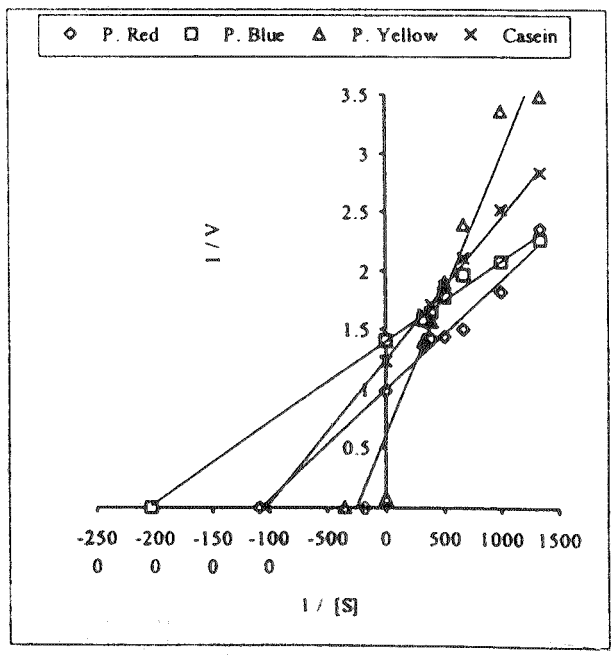

Fig. 16. Appropriate chymotrypsin concentration with procion yellow-casein complex

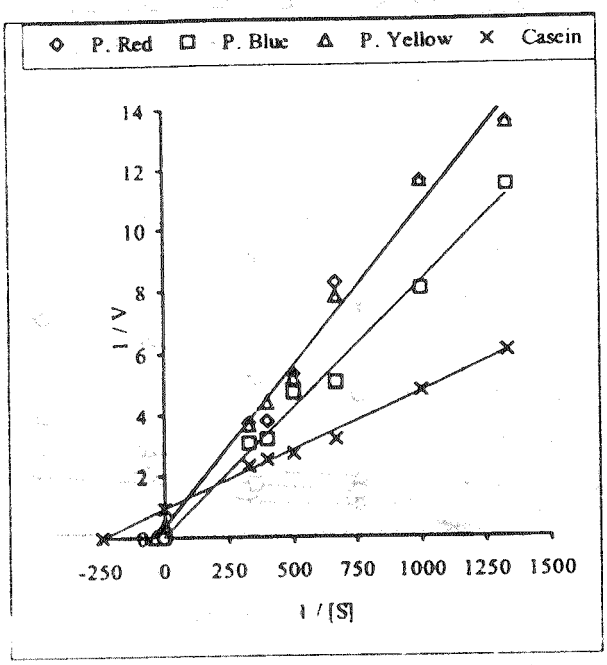

Fig. 17. Lineweaver-Burk plots of subtilisin carlsberg with casein, natural dyes-casein complexes

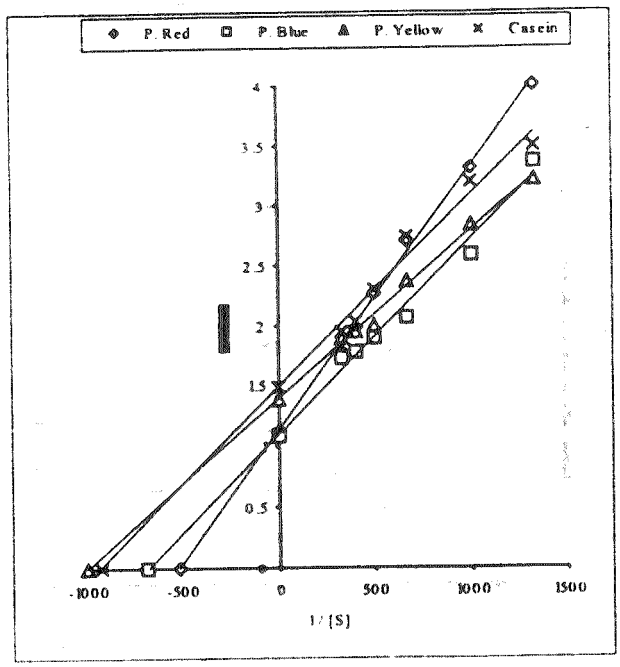

Fig. 18. Lineweaver-Burk plots of protease type XVI with casein, synthetic dyes-casein complexes

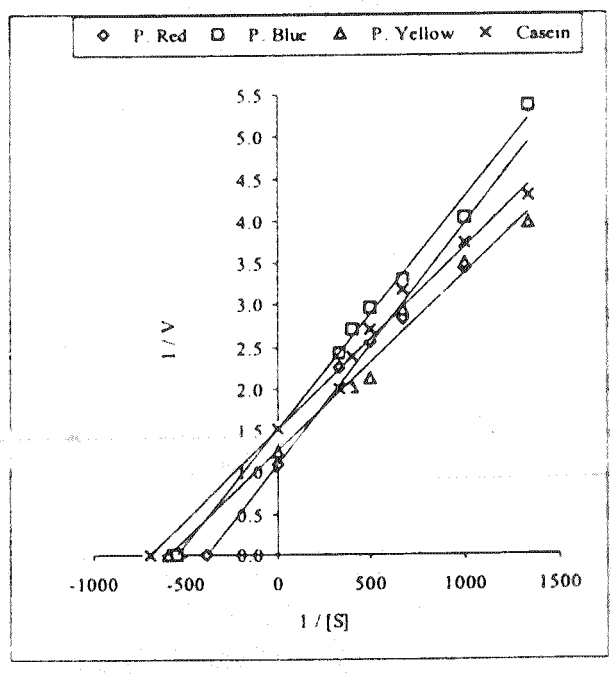

Fig. 19. Lineweaver-Burk plots of trypsin with casein, synthetic dyes-casein complexes

has been studied. The maximum activity providing pH values for these enzymes has been determined. Also the enzyme amount and incubation time for each enzyme at which the reaction rate is in primary degree was established. The results are shown in graphics, Figs. 1–16, and then the changes in catalytic activity with increasing concentration of casein staining material prepared at optimal conditions were studied. The Lineweaver-Burk conversion of obtained data is shown in graphics, Figs. 17–19. Although kinetic contants are not routinely calculated when large molecules are used as substrate, we have studied K_m, V_{max}, k_{cat} and (k_{cat}/K_m) values to get an opinion (Tables 1, 2).

TABLE-1
K_m, V_{max}, k_{cat} AND k_{cat}/K_m VALUES OF SUBTILISIN CARLSBERG AND PROTEASE TYPE XVI

Substrates (Casein-dye complexes)	Subtilisin Carlsberg			Protease type XVI		
	K_m (g/mL)	V_{max} $(M.s^{-1}) \times 10^{-2}$	k_{cat}/K_M (mL/g.s)	K_m (g/mL)	V_{max} $(M.s^{-1}) \times 10^{-2}$	k_{cat}/K_M (mL/g.s)
Procion red	9.2×10^{-4}	1.019	150	3.8×10^{-2}	3.586	8
Procion blue	4.9×10^{-4}	0.706	190	2.1×10^{-1}	25	10
Procion yellow	2.8×10^{-3}	1.893	600	2.7×10^{-2}	2.616	8
Casein	9.7×10^{-4}	0.807	110	4.2×1^{-3}	1.095	23

TABLE-2
K_m, V_{max}, k_{cat} AND k_{cat}/K_m VALUES OF TRYPSIN AND CHYMOTRYPSIN

Substrates (Casein-dye complexes)	Trypsin			Chymotrypsin		
	K_m (g/mL)	V_{max} $(M.s^{-1}) \times 10^{-2}$	k_{cat}/K_M (mL/g.s)	K_m (g/mL)	V_{max} $(M.s^{-1}) \times 10^{-2}$	k_{cat}/K_M (mL/g.s)
Procion Red	2.0×10^{-3}	0.888	60	2.6×10^{-3}	0.910	170
Procion Blue	1.5×10^{-3}	0.916	80	1.8×10^{-3}	0.658	180
Procion Yellow	1.0×10^{-3}	0.716	90	1.6×10^{-3}	0.802	250
Casein	1.1×10^{-3}	0.672	80	1.5×10^{-3}	0.658	230

* Following wavelengths were used for all experimental conditions:
$\lambda_{jugkin\text{-}casein}$: 396 nm; $\lambda_{lawsone\text{-}casein}$: 453 nm; $\lambda_{berberine\text{-}casein}$: 346 nm; $\lambda_{quercetine\text{-}casein}$: 346 nm

The substrate appropriateness of synthetic staining substances was arranged from procion yellow to procion red and finally procion yellow as may be supposed from K_m and k_{cat}/K_M values. The closeness of catalytic activity of trypsin and chymotrypsin on these substrates once again shows that these are in the same group of enzymes.

In conclusion, all prepared complexes were found to be appropriate substrates for enzymes used.

While substrate fitness of prepared proteolytic enzymes was being studied, the appropriate pH, incubation time, agitation speed like parameters have been studied for each substrate and enzyme. Therefore for each substrate and enzyme

an upper activity level has been determined. Even under these circumstances the activity of enzymes to each substrate has been found to be significantly different.

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