

Study of Enzyme Activity for Pepsin and its Complexation with Dexamethasone and their Characterization

S. Saeed-ul-Hassan,* F.Z Khan and F. Deebea

Faculty of Pharmacy, Pharmaceutical and Phyto Chemistry Group, University Of The Punjab Allama Iqbal Campus, Lahore-54000, Pakistan.

Abstract

The enzyme activities for pepsin and its complexation with dexamethasone were carried out. The pepsin activity both in free and complex form was studied by spectro fluorimeter using a newly prepared fluorosubstrate and proved to show the enzyme activity. The immunoreactivity of dexamethasone-Pepsin complex was also studied by rapid enzyme linked immunosorbent (ELISA) assay which proved that this complex was immunoreactive to dexamethasone specific antibodies. These studies will be useful for the preparation of a variety of drug-enzyme complexes.

Keywords: Pepsin, Dexamethasone, Complexes, ELISA.

Introduction

Pepsin is a proteolytic enzyme found in the gastric and other tissues of different animals like cattle, sheep swine, rabbit, buffalo, chicken, ducks, camel and monkey (Saeed *et al.*, 1999). It has also been reported that the young animals have more pepsin in their gastric tissues than old animals. Pepsin is most active at a pH range of 1.8-2.5 whereas entirely inactive in neutral or alkaline media. It converts proteins into proteoses and peptones (Varro *et al.*, 1981). Pepsin has many useful applications. It has been used as a digestive enzyme in many pharmaceutical preparations. Pepsin has also many useful industrial applications such as making cheese and protein hydrolysates (Saeed *et al.*, 1999). Dexamethasone is a synthetic gluco-corticoid which is widely used as human and veterinary medicine. It is a potent anti-inflammatory agent and has become a popular alternative to proper rest to keep horses in training. We have reported previously the enzyme linked immunosorbent assay (ELISA) for dexamethasone in equine urine by colorimetric (Hassan *et al.*, 1997) fluorescent (Hassan *et al.*, 1996) and DOT ELISA methods (Hassan *et al.*, 1998). Drug enzyme conjugate of dexamethasone - subtilisin and dexamethasone - cellulase have been synthesized and their characterization with respect to their drug protein incorporation ratio, immuno reactivity, enzyme activity and stability have been described by Hassan (Hassan *et al.*, 2000). A detailed review of analytical methods for dexamethasone has also been described (Hassan *et al.*, 2001a and 2001b). The main aim of this work was to prepare the dexamethasone-pepsin (drug-enzyme) complex to study the enzyme activity of pepsin in free and bound (complex) form and further to investigate the immunoreactivity of this complex as a proof for our previously published work (Hassan *et al.*, 2000) for the preparation of a variety of drug-enzyme complexes (conjugates).

* Corresponding author E-mail: nizamani_19@hotmail.com

Material and Methods

Apparatus: Perkin-Elmer Luminescence spectrometer Model LS 50B was used and the results were continuously logged into and processed by dedicated PC with Perkin Elmer FL data manager software installed. The size exclusion chromatography system composed of a 9 ml Sephadex G-25 M Column (PD-10), a peristaltic pump (P-1), a single path monitor and its control units (UV-1), a fraction collector (Frac 100) and a chart recorder. These components were all obtained from Pharmacia (Milton Keynes) Buckinghamshire (U.K). The option 3 water purification system was purchased from Elga, U.K and pH meter was model 3020 (Jenway Ltd, U.K).

Reagents: Glycine buffer (0.1M) was prepared by dissolving 7.5g of glycine powder in 1L distilled water and adjusting pH to 2.3 with dil HCL. Lucifer yellow dilithium salt, tetramethylrhodamine isothiocyanate (TRITIC), bovine serum albumin (BSA) pepsin, 1-ethyl-(3-dimethyl-amino propyl) carbodiimide hydrochloride (EDC) and dexamethasone were purchased from Sigma chemical (Poole, Dorset, U.K). All the buffer salts were obtained from BDH and were of analytical reagent grade. Sheep antidexamethasone antiserum AD61 was supplied by the Horse Racing Forensic Laboratory, Suffolk, U.K.

(I) Preparation of double labelled substrate for pepsin activity: The method employs a bovine serum albumin substrate labelled with two fluorescent dyes with fluorescence energy transfer (FET) characteristics. The double labelled substrate was prepared by chemically coupling bovine serum albumin with Lucifer yellow (LY) and rhodamine (Rh) dyes according to Tang *et al.*, 1995a. The fluorescence emission from the Lucifer labels was initially quenched due to FET of LY fluorescence to the adjacent Rh labels. Upon the addition of Pepsin to the labelled substrate solution, increased fluorescence was observed as the enzyme hydrolyzed the substrate, generating small fragments in which only one fluorophore was present. Hence enhancement of the LY versus fluorescence was observed as the FET effect was reduced. For the synthesis of double labelled fluorosubstrate, the reaction mixture (75 mg BSA, 6.5 mg Ly and 5mg TRITC) was purified by size exclusion chromatography system (David *et al.*, 2005). The fractions collected from the first peak were pooled together, diluted (1:10) in glycine buffer (pH 2.3) and stored at 4°C until used for the assay of pepsin activity (Tang *et al.*, 1995b and 1996).

(II) Assessment of pepsin activity by spectro fluorimeter: A standard dilution of pepsin enzyme ($20 \mu\text{g ml}^{-1}$) was prepared in glycine buffer (pH 2.3) and from this dilution 10 μl . equivalent to 200 nanogram of pepsin was added into a fluorescence microcell, followed by the addition of 590 μl of substrate solution (prepared as above (I) to make the final volume of 600 μl into the micro cell) and the fluorescence reading was immediately started. The excitation and emission of wavelengths selected were 430 and 535 nm, with the slitwidths of 8.0 and 10.0 nm using Perkin-Elmer luminescence spectrometer (model LS 50B, Perkin-Elmer). A mixture of simple glycine buffer (10 μl) and substrate solution (590 μl) to make the final volume of 600 μl into a microcell was also used as a blank to record the fluorescence intensity units. The results are shown in Figs. 1 and 2.

(III) Preparation of dexamethasone-pepsin complex: A complex of dexamethasone hemisuccinate-pepsin (dex-pepsin) using 1:10 molar ratio of enzyme drug was prepared. 10 mg of dexamethasone hemisuccinate, 4 mg of EDC (1-ethyl-3-dimethyl amino propyl) carbodiimide hydrochloride) and 2.5 ml of dry pyridine into a small quickfit flask and the reaction was carried out in fuming cupboard at room temperature for about 1 h using magnetic stirrer, after that 70 mg of pepsin previously dissolved in 10 ml of distilled water was added to

the above reaction mixture and the reaction was carried out overnight in a fuming hood at low speed to complete the reaction and this reaction mixture was transferred into a dialysis tubing and dialysis was carried out against distilled water for few hours until removing the smell of pyridine and finally the reaction mixture was subjected to size exclusion chromatography to obtain the purified drug-pepsin complex (conjugate).

(IV) *Purification of drug-enzyme complex by size exclusion chromatography:* A 9 ml column (PD-10) of Sephadex G-25 M was equilibrated with distilled water using a flow rate of 1 ml/min, a chart speed of 5mm/min. set at 100x2mv sensitivity and a 280 nm detector (David *et al.*, 2005). The reaction mixture as obtained by dialysis was passed separately through this column in 1 ml aliquots. The eluted fractions were stored at 4°C until used.

(V) *Assessment of enzyme activity of pepsin-dexamethasone complex:* The enzyme activity of the eluted fraction (pooled) was carried out as described above (II) after the preparation of exactly the same dilution in glycine buffer as did for the standard pepsin. The result is shown in Fig.3.

(VI) *Determination of the immunoreactivity of the drug-enzyme complex:* A separate dexamethasone enzyme linked immunosorbent assay (ELISA) was carried out to assess the immuno reactivity of the dexamethasone - pepsin complex by using the standard colorimetric single reagent ELISA as described by Hassan *et al.*, 1997, using 50 µl aliquots of diluted drug-enzyme complex in assay buffer to run as a samples. The optical density values for standards of dexamethasone and sample of enzyme-complex are given in the Table 1.

Table 1. Colorimetric determination of dexamethasone and dexamethasone-pepsin complex by rapid ELISA.

Standard curve				Dex-Pepsin Fraction			
Conc. of Dex(M)	O.D (n=6)	SD	CV (%)	Dilution of fraction	OD (n=6)	SD	CV
Blank (Zero)	1.146	0.025	2.2	1:10 ³	0.373	0.008	2.2
1x10 ⁻⁹	1.078	0.047	4.4	1:10 ⁴	0.934	0.030	3.6
5x10 ⁻⁹	0.983	0.042	4.3	1:10 ⁵	1.057	0.030	3.0
1x10 ⁻⁸	0.885	0.036	4.0	1:10 ⁶	1.128	0.030	3.1
5x10 ⁻⁸	0.455	0.011	2.4	-	-	-	-
1x10 ⁻⁷	0.430	0.022	5.1	-	-	-	-
Buffer only	0.065	0.001	1.7	-	-	-	-

Results and Discussion

The enzyme activity for Pepsin was carried out by using the double labelled substrate. Fig 1 shows the increase in fluorescence intensity units (40 units) with respect to background signals which indicate that the standard enzyme (Pepsin) showed pronounced activity in glycine buffer at pH 2.3, as this enzyme had a proteolytic activity at acidic pH. A mixture of simple glycine buffer (without enzyme) and substrate solution was also used as a blank to make the comparison of increase in fluorescence units.

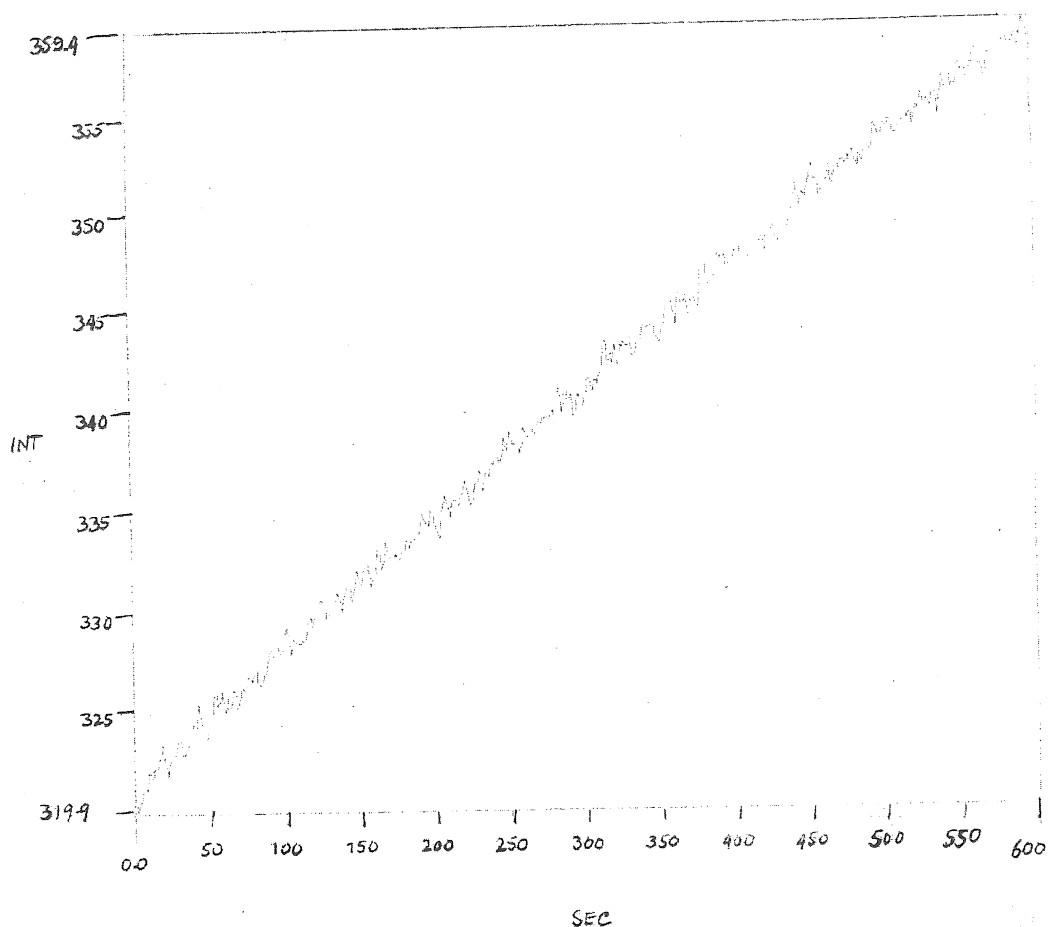


Fig. 1. Study of enzyme activity for pepsin versus time (seconds) and fluorescence intensity using double labeled substrate by spectrofluorimeter.

Fig. 2 shows only the increase in 0.6 fluorescence units which indicates the absence of enzyme. Therefore Figs 1 and 2 indicate the clear cut difference between the standard pepsin and blank pepsin. It is interesting to note that when the basic reagent (e.g diethylamine, triethylamine, cystine + EDTA) was added in turn to the substrate solution to study the pepsin activity, the enzyme was deactivated and no increase in fluorescence units was observed (data not shown) which has proved that the substrate solution worked very well in acidic media.

The enzyme activity for dexamethasone-pepsin complex was also studied by using the same substrate as used for pepsin standard enzyme. Fig.3 shows the 15 units increase in the fluorescence intensity indicating about 70% reduction in signal as compared to standard pepsin (Fig. 1). This proved that pepsin was still active in the complex form as it showed the noisy peaks as compared with the standard pepsin peaks.

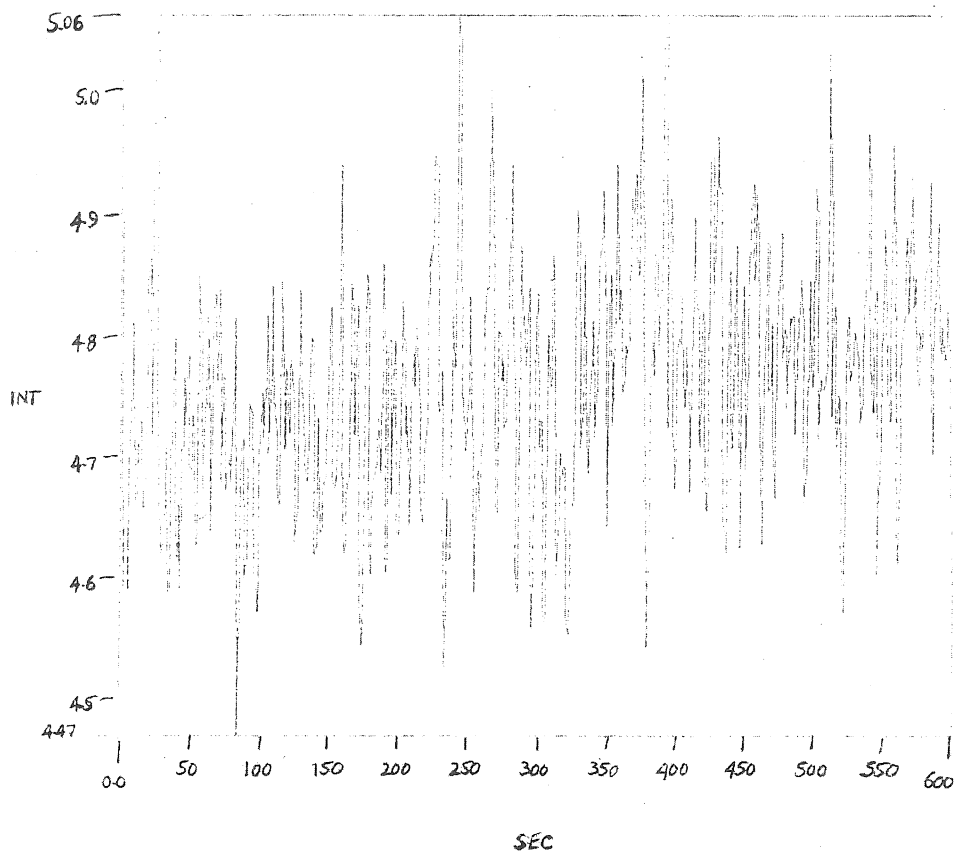


Fig. 2. Study of enzyme activity as a blank versus time (seconds) and fluorescence intensity using glycine buffer and doubly labeled substrate by spectrofluorimeter.

The immunoreactivity of the dexamethasone pepsin complex was also studied by simple single reagent ELISA method. The results in Table 1 showed that this complex was immunoreactive to the dexamethasone specific antibodies. The incorporation ratio for dexamethasone-pepsin was interpolated from the standard curve (Table 1) using the O.D values of the respective complex and 2.6:1 (Dex-Pepsin) ratio was found which still proved its activity in terms of complexation and stability.

It is concluded from the above discussion that the drug-enzyme conjugate retain their immunoreactivity as well as enzyme activity in the complex form, indicating that these studies are useful for the synthesis of a variety of drug-labelled conjugates as previously proved as one of the novel example for the synthesis of dexamethasone-subtilisin and dexamethasone-cellulase conjugate (Hassan *et al.*, 2000)

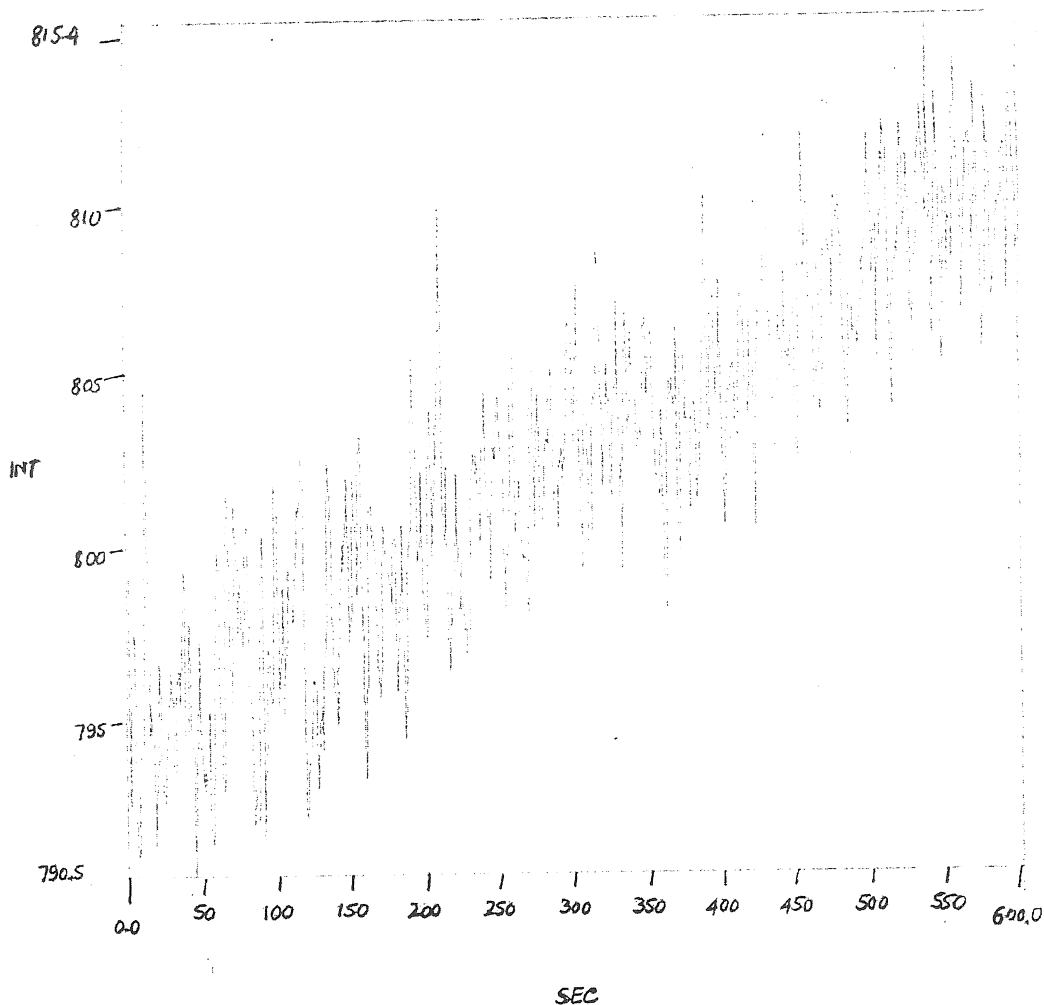


Fig. 3. Study of enzyme activity for ex-pepsin complex versus time (seconds) and fluorescence intensity using doubly labeled substrate by spectrofluorimeter.

References

David, L.N., Michael, M.C (2005). Lehninger Principles of Biochemistry, 4th Edition W.H. Freeman and company New York.

Hassan, S.S, Rowell, F.J. and Jackson, L.S. (1996). Rapid fluorescence enzyme linked immunosorbent assay screen for dexamethasone in equine urine. *Ann. Commun.* 33: 299- 302.

Hassan, S.S., Rowell, F.J. and Jackson, L.S. (1997). Enzyme linked immunosorbent assay for dexamethasone in equine urine. *Pak. J. Med. Res* 36: 75- 78.

Hassan, S.S., Rowell, F.J. Hambleton, P., Jackson, L.S. (1998). A rapid on filter immune assay screen for dexamethasone in equine urine. *Ann. Commun* 33: 249 – 252.

Hassan, S.S. and Rowell, F.J. (2000). Synthesis and characterization of drug enzyme conjugates. *Arch. Pharm Res.* 23: 548- 553.

Hassan, S.S., Rowell, F.J. and Jackson L.S., (2001a). Analytical methods for dexamethasone. *Acta Pharm. Turcica* 43: 33 – 42.

Hassan, S.S., Rowell F.J. and Jackson, L.S. (2001b). Effect of affinity purification on the sensitivity and precision of a simplified rapid enzyme linked immunosorbent (ELISA) assay for dexamethasone. *Acta Pharm. Turcica* 43: 51-58.

Saeed, A.M., Raffique, F.M. (1999). Isolation of Pepsin from Buffalo and its activity. *Acta Pharm. Turcica.* 41: 181 – 185.

Tang, L.X., Rowell, F.J., Cumming, R.H. (1995a). Rapid and sensitive assay for some protease enzymes using a fluoro substrate – immobilized. *Bioreactor.Analyst* 120: 1949 – 1952.

Tang, L.X., Rowell, F.J. and Cumming, R.H. (1995b). Ultra- rapid fluorescence assay for cellulase using a substrate immobilized minibioreactor. *Anal. Proc. Includ. Ann. Commun.* 32: 519 -520.

Tang, L.X., Rowell, F.J., Cumming, R.H. (1996). A Rapid Homogeneous fluorescence assay for subtilisin. *Anal. Lett* 29: 2085 – 2095.

Tang, L.X., Rowell, F.J., Cumming, R.H. (1996). Development of near real time monitoring systems for some serine protease enzymes in the industrial atmosphere. *Ann.. Occup. Hyg.* 40: 381 – 389.

Varro, E.T., Lynn R.B., James, E.R (1981). Pharmacognosy, 8th Edition Lea and Febiger Philadelphia.

Received: 29.03.2002

Accepted: 26.10.2004