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PREPARATION OF SUBTILISIN COMPLEXES WITH ALPHA-2-MACROGLOBULIN AND THEIR CHARACTERIZATION

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خلقت متراكبات مع صبتلزين مع دكساميثازون ، وألفا ٢ ماكروجلوبيولين ، وقد وجد أن تلك المتراكبات لها خاصية نشاط مناعى ضد مضادات دكساميثازون ، كما أن هذه المتراكبات تقلل من التحلل البريتونى عندما نستعمل منها ذات الوزن الجزئى القليل والذى يستخدم بطرق القياس الضوئى المتجانس. كما إستخدمت بعض منها ذات دلالات لدراسة نشاط الانزيمات فى هذه المتراكبات. كما أن دكس صبتلزين له خاصية تحلل بروتونى علما بأن دكس صبتلزين أم ليس له خاصية التحلل البروتونى وذلك لأنه لا يحتوى على زيادة من المركبات ذات الوزن الجزئى الصغير على المركبات ذات الوزن الجزئى الكبير من البروتينات فى الخليط.

The subtilisin complexes with dexamethasone and Alpha-2 macroglobulin were synthesized and were found to be immunoreactive to the specific antidexamethasone antibodies. These complexes also retain proteolytic activity when low molecular weight substrate was used by Homogeneous colorimetric method. The doubly labelled substrate was also used to study the enzyme activity in complex form. The Dex-subtilisin showed the proteolytic activity whereas Dex-subtilisin A_2M did not show the proteolytic activity due to no access of small molecular weight substrate towards the large molecular weight protein in a co-complex form.

INTRODUCTION

Preparation of drug-enzyme conjugates are important to study enzyme activity,immuno-reactivity, protein incorporation ratio, and stability. We have reported the synthesis and characterization of Dexamethasone-subtilisin and dexamethasone-cellulase complex[1] and we have also reported the preparation of Dexamethasone-Pepsin complex and its characterization.[2] Alpha-2 macroglobulin (A_2M) is one of the major plasma proteinase regulators in man and reacts with a wide variety of endopeptidases.[3-4] After enzymatic cleavage in a well defined area of the molecule called the bait region. A_2M undergoes a change of conformation entrapping the proteinase inside the molecule. Enzymatic inhibition in the resulting complex with large substrates is due to the steric hindrance-however the active site of the enzyme keeps its function and hydrolysis of small substrate is only slightly impaired.

A similar change in conformation occurs after treatment with nucleophiles such as methyl amine.[5-6] A_2M is the major representative of a group of plasma proteins that include complement components C_3 and C_4 as well as pregnancy zone protein (PZP). This protein family is collectively called the Alpha Macroglobulins. Because these proteins have a unique internal cyclic Thiolester bond, they have also been termed the Thiolester plasma protein family.[7] Several models differing in symmetry and localization of the disulfide-bridged dimers have appeared. The model shown in Fig. 1. proposes that in native tetrameric human A_2M, two rod like disulfied-bridged dimers are assembled to form a cross-like structure. The dimers presumably constitute its functional proteinase binding units. This model accounts for the generally elongated shape of the dimers and monomers and emphasizes that in the native state the bait region segments are very accessible to proteinases.[8] Since the Alpha-2 Macroglobulin (A_2M) binds, it may be assumed that A_2M will also bind to dexamethasone-subtilisin complex (Dex-Sub-A_2M) and this conjugate will retain its proteolytic activity and immuno reactivity.

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We have also reported the enzyme linked immunosorbent assay (ELISA) for dexamethasone by colorimetric,[9] Fluorescent[10] and DOT ELISA[11] techniques and have also described a detailed review of analytical methods for dexamethasone in biological fluids.[12]

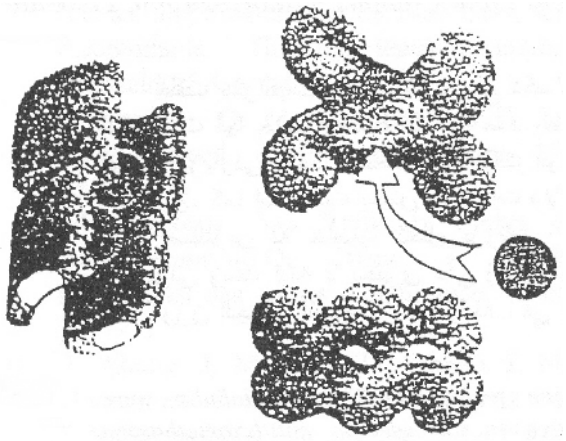

Fig. 1: Model of native human alpha-2-macroglobulin and proteinase complexes.

MATERIAL AND METHOD

Apparatus

Dynatech Immulon 4 microtitre Plates (Dynatech Laboratories, West Sussex, U.K) Dynatech MR 700 Plate reader, Eurotherm(Electric oven) Model 8-300 was purchased from Townson and Mercer,U.K. Perkin Elmer Luminescence spectrometer LS 50B- with Epson LX-1050 Printer. The option 3 water purification system was purchased from Elga, U.K and pH meter model 3020 (from Jenway Ltd, U.K).

Reagents

N-Succinyl-ala-ala-Pro-Phe-Paranitro-anilide (MW=624) A_2-macroglobulin from human plasma (molwt 725,000/- subtilisin carlsberg (protease type VIII) from bacillus Licheniformis (Molwt 27,500) Dexamethason, Lucifer yellow dilithium salt (molwt 550) tetramethylrhodamine isothiocyanate TRITC (MW= 480) were purchased from the sigma chemical company (Poole, Dorset, U.K). All reagents were prepared using ultra pure water (Elga Water Purifier system) and antidexamethasone antiserum AD61 was supplied by the Horse racing Forensic Laboratory Suffolk,U.K. All other Buffer salts and reagents were of analytice grade.

Experimental

i) Preparation of subtilisin complexes with alpha-2-macroglobulin (A_2M)

The dexamethasone-subtilisin (Dex-Sub) complex was prepared according to the method described elsewhere[1] and this complex was used to make the co-complex with $Alpha_2$ Macroglobulin (Dex-Sub-A_2M) using 1:1 molar ratio Dex-subtilisin complex with A_2M using phosphate buffer saline with tween (PBST) at pH 7.4.

ii) Study of the immuno reactivity of Dex-Sub and Dex-Sub-A_2M complex by two step ELISA for dexamethasone

The immuno reactivity of the complexes were carried as following the published two step Enzyme-linked immunosorbent assay (ELISA) as briefly shown in Fig. 2 for dexamethasone[9]. The buffer used in this study was Phosphate buffer saline with tween (PBST) + 0.1% Bovine serum albumin (BSA) at pH 7.4 to block possible digestion of the Dexamethasone Porcelin Thyroglobulin (Dex-PTG) plate coating by subtilisin. The samples used in this format were Dex-subtilisin and Dex-subtilisn A_2M. These were diluted in the buffer to give concentration equivalent to 100 ng ml^{-1} of the enzyme activity. These samples were tested as an unknown on the same microtitre plate which was also used to run the dexamethasone standards. The results for dexamethasone standards and for the complexes are given in Table 1.

iii) Assessment of the enzyme activity of Dex-Sub-A_2M by using n-succinyl-ala-ala-Pro-Phe-p-nitroanilide SAA-pNA)

The enzyme activity for A_2M-Dex-Sub (1:1 molar ratio) was carried out by using the homogeneous colorimetric method[1]. Aliquots of 50 µl of he diluted complex were added to the wells of he microtitre plate followed by 50 µl of 1 mM SAA-pNA substrate to one column of the plate. To the other column 50 µl of buffer (0.1M Tris buffer + 0.1% Tween pH 8.6) were added followed by 50 µl of substrate. After an interval of 30 min, the absorbance of the solution was measured at 405 nm. and the results are given in Table 2 and Fig. 3.

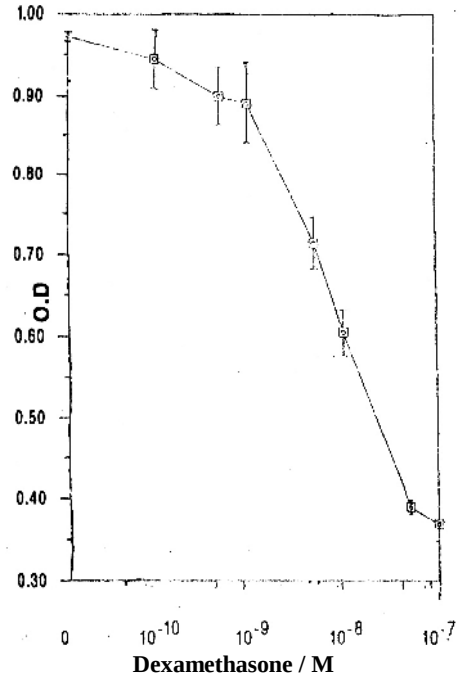

Fig. 2: Standard curve for dexamethasone by colorimetric method (mean ± 1s, n= 5).

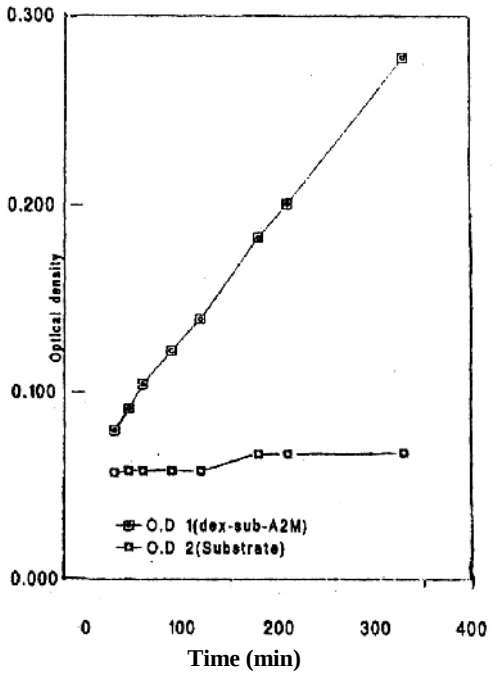

Fig. 3: Study of the enzyme activity of the Dex-Sub-A_2M by homogeneous colorimetric method at 405 nm.

Table 1: Average optical density values for standards of dexamethasone and complexes by two step colorimetric ELISA.

OD for standard curve				OD for complexes	
Conc. of DEX (Mol/l)	O.D (n=6)	SD	CV (%)	Dex-sub (n=6)	A2M-Dex-sub (n=6)
Blank (0)	1.090	0.036	3.30	OD=0.364	OD=0.436
$1.0x10^{-9}$	1.000	0.030	3.00	SD=0.017	SD=0.018
$5.0x10^{-9}$	0.710	0020	2.81	CV=4.67	CV=4.12
$1.0x10^{-8}$	0.550	0.011	2.00		
$5.0x10^{-8}$	0.310	0.010	3.22		
$1.0x10^{-7}$	0.237	0.007	2.95		
$1.0x10^{-6}$	0.163	0.007	4.29		

Table 2: Enzyme activity of the Dex-Sub-A_2M using N-succeinyl-ala-ala-Pro-Phe-pnitroanilide (SAA-pNA)substrate.

Time (min)	Dex-Sub-A_2M O.D (n=4)	S.D	CV %	Substrate only OD (n-4)	S.D	CV %
30	0.079	0.001	1.26	0.056	0.001	1.78
45	0.091	0.001	1.09	0.057	0.001	1.75
60	0.104	0.001	0.96	0.057	0.001	1.75
90	0.122	0.001	0.0	0.057	0.001	1.75
120	0.139	0.001	0.71	0.057	0.001	1.75
180	0.183	0.001	0.54	0.066	0.002	3.03
210	0.201	0.002	0.99	0.066	0.002	3.03
330	0.278	0.001	0.35	0.067	0.002	3.03

iv) Assessment of enzyme activity of Dex-Sub- A_2M using doubly-labelled fluoro substrate by homogeneous fluorescence method

The method was followed as reported in the literature.[2] Two samples, Dex-subtilisin and Dex-subtilisin A_2M were tested by taking the 10µl volume (equivalent to 200 ng of subtilisin) of the sample into the micro cell followed by the fluoro-BSA (bovine serum albumin labelled with lucifer yellow and rhodamine dyes) solution to make the final valume 600 µl. After mixing, the fluorescence reading was immediately started using a Perkin-Elmer luminescence spectrometer. The excitation and emission wavelengths of the fluorescence detector were set at 430 and 535 nm, respectively both with slit widths of 8 nm. The total assay time for both the samples were 10 and 166 minutes respectively and the results are shown in Figs. 4-6.

RESULTS AND DISCUSSION

The results in Table 1 indicates that the Dex-substilisin and Dex-subtilisin A_2M are immuno reactive to the specific antidexmethasone antibodies. The concentration of dexa methasone in these complexes were interpolated from the standard curve and their molar ratio were calculated as 12.3:1 and 7.7:1 for dex:sub and Dex-sub: A_2M complexes respectively.

The results in Table 2 indicates that this complex retain its proteolytic activity towards the p-nitroanilide substrate which has a low molecular weight (MW= 624.6) but the activity of this complex is significantly reduced in the complex form as compared to the untreated subtilisin and Dex-subtilisin[1]. Therefore, this experiment conforms that low molecular weight substrate can gain access to the active site within the Dex-Sub A_2M complex so that the complex retains its proteolytic activity.

The results in Fig. 4 indicates an increase of 20 fluorescnece units over 10 minutes time in the presence of Dex-subtilisin using the doubly labelled fluoro substrate. In contrast with Dex-Sub A_2M under the same conditions, no increase in fluorescence intensity was observed as shown in Fig. 5. Increasing the incubation time from 10 minutes to 166 minutes still showed no increase in signals as also shown in Fig. 6. This shows that a large substrate (RMM= 69030) can not gain access to the subtilisin within the complex. This is in contrast to the retention of activity observed for the p-nitroanilide peptide substrate (RMM= 624.6). It can also be concluded that the enzyme is completely associated with the complex and hence this experiment also confirms the electrophoresis results (data not shown here) for the A_2M-Dex-Sub complex which indicates this situation. These results confirms literature observations for A_2M proteolytic enzyme complexes.[3]

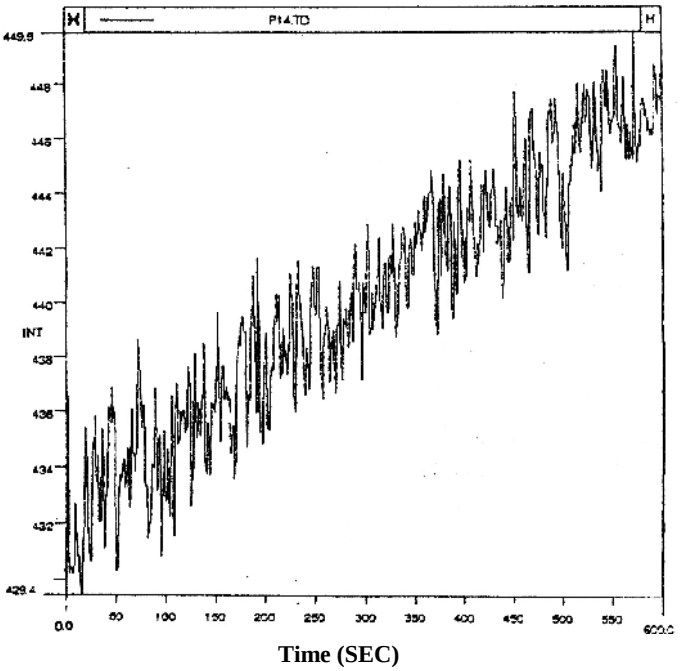

Fig. 4: Study of enzyme activity of Dex-Sub complex using doubly labelled fluorosubstrate by homogeneous fluorescence method.

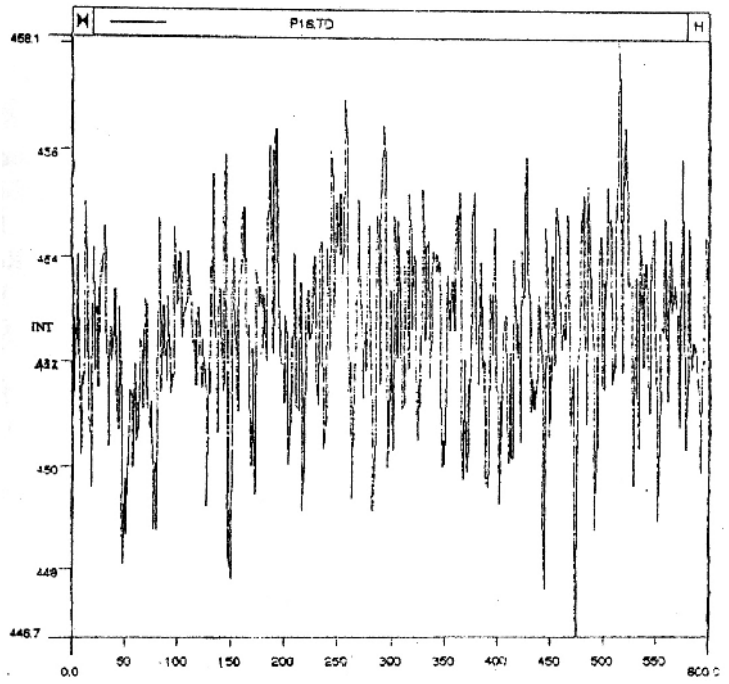

Fig. 5: Study of enzyme activity of Dex-Sub-A_2M complex using doubly labelled fluorosubstrate by homogeneous fluorescence method (10 min).

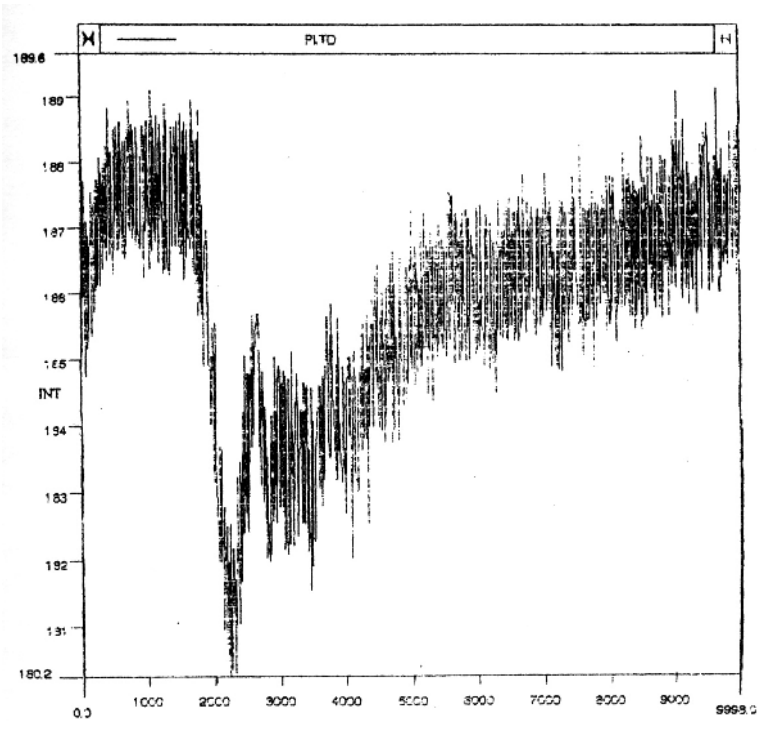

Fig. 6: Study of enzyme activity of Dex-Sub-A_2M complex using doubly labelled fluorosubstrate by homogeneous fluorescence method (166 min).

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