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Characterization of chemically modified avian ovomucoid III domain

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ABSTRACT

Protease-antiprotease balance is involved in a number of biological and pathological processes and in egg white, proteases are thought to have role in embryogenesis and antimicrobial defense. Other biological activities associated with egg white include immunomodulatory, anti- cancer, antioxidant and anti-hypertensive properties. The avian egg is an important source of nutrients, containing all of the proteins, lipids, vitamins, minerals and growth factors required by the developing embryo Ovomucoid, a serine protease inhibitor, is useful in oral delivery of protein/ peptide therapeutics. Co-administration of ovomucoid with calcitonin, is often used in the management of osteoporosis. Because ovomucoid inhibits digestive enzymes, such as trypsin, a- chymotrypsin and elastase, it has been found to improve the oral delivery of insulin. In view of its various important roles, ovomucoid was isolated from duck and its third domain was characterized. The present study highlights the characterization of chemically modified third domain.

INTRODUCTION

Ovomucoid is a serine proteinase inhibitor in the egg whites of all avian species at a concentration of about 10 mg/ml which accounts for 10% of the protein produced by the tubular gland cells of the oviduct in laying birds[1-7]. The involvement of proteinases in a multitude of control functions in an organism has created an interest in their physiological inhibitors. Regulation of proteolytic activity in tissues is a critical requirement in the maintenance of homeostasis[8-13]. Egg white proteins possess ACE-inhibitory activity, & also high radical-scavenging activity. The combined antioxidant and ACE-inhibitory properties of egg white hydrolysates, or the corresponding peptides, would make a useful multifunctional preparation for the control of cardiovascular diseases[14-20]. Egg proteins are rich source of biologically active peptides. Besides having protease inhibitory activity, several other biological activities are associated with egg components, including novel antimicrobial activities, ant adhesive properties, immunomodulatory, anticancer, antioxidant, antihypertensive properties.[21-25] Proteases play key roles in several physiological processes, including intracellular protein degradation, bone remodeling, and antigen presentation, and their activities are increased in pathophysiological conditions such as cancer metastasis and inflammation. They are also required for invasion by microorganism. Four protease inhibitors have been identified in egg white: cystatin, ovomucoid, ovomacroglobulin (also known as ovostatin), and ovoinhibitor[26-28]. Ovomucoid-a serine proteinase inhibitor has been found to be useful for oral delivery of

protein/peptide therapeutics[29-34]. Avian egg whites are a rich source of protein inhibitors of proteinases belonging to all four mechanistic classes. Ovomucoid and ovoinhibitor are multidomain Kazal-type inhibitors with each domain containing an actual or putative reactive site for a serine proteinase[35].

Laskowski et al[36]. have reviewed the medicinal and biological aspects of proteinase inhibitors. The possible use of proteinase inhibitors in the treatment of various diseases which include cancer and Aids, has led to extensive studies on the structure, specificity and stability of these inhibitors. To be able to increase the activity and/or stability of a protein, the knowledge of relationship between structure and stability of the molecule is necessary. It has been found that globular proteins with hydrophobic and compact interiors are more stable as compared to those having polar or charged amino acid residues in their interior[37].

The third domain of duck ovomucoid (OMDUK III) is a globular protein with three lysine residues, two of which have less reactivity and are probably shielded from the environment by neighboring amino acid residues. In this study, chemical modification of the three lysine residues present in the third domain of duck ovomucoid was carried out.

The aim was to find out if the chemical modification of one or more lysine residues affects the stability of the domain, its interaction with the enzyme in the enzyme-inhibitor complex formation, or alters the specificity of OMDUK III towards any enzyme.

MATERIALS AND METHODS

Isolation of duck ovomucoid

Sephacryl S-100HR column (1.938x1.83.4cm) was used for the purification and the purity was checked by gel filtration,SDS-PAGE (sodium dodecyl sulphate – polyacrylamide gel electrophoresis),and fluorescence spectroscopy[38].

Isolation and characterization of third domain of duck ovomucoid

Cleavage of ovomucoid with theremolysin was performed in 0.2 M Tris-Hcl buffer, pH 8.1 according to the method described by Laskowski et al[39]. The third domain was separated on a Sephacryl S-100HR column (1.938x1.83.4cm)

Chemical modification of third domain of duck ovomucoid

The chemical modification of lysine residues of duck ovomucoid third domain (OMDUK III) was carried out. Using three different modifying reagents: acetic ahydride (A) (which neutralizes the positive charge of lysine), succinic anhydride (S) (which replaces the positive charge by negative), and o-methyl isourea (G)(which retains the positive charge but increases the size of the residue).

To determine the extent of modification achieved by different amounts of modifying reagent used, the percent modification achieved was plotted against the molar excess of modified reagent taken over protein. for this purpose, identical amounts of OMDUK III were treated with varying molar excess of acetic anhydride. the molar excesses giving 33%, 67%, and 100 % modifications were subsequently taken with the three modifying reagents to modify one, two or all three lysine residues of the protein.

A1, S1 and G1 corresponds to 33% modifications; A2, S2 and G2 corresponds to 67% modification;A3, S3 and G3 corresponds to 100% modification.

Acetylation of OMDUK III

This was carried out according to the method described by Riordan and Vallee (1967)[40] using acetic anhydride as the acetylating agent. The protein was taken in 0.2 M sodium phosphate buffer, pH 7.5, and the required amount of acetic anhydride was added slowly with continuous stirring. The reaction was performed at 4^{o} C and the pH of the reaction was maintained between 7.0 and 7.8 by appropriate additions of 2 M NaOH (sodium hydroxide). The reaction was continued until pH changes stopped (about one hour)

Deacetylation of acetylated tyrosine residues: Acetic anhydride is not absolutely specific for lysine residues, tyrosine residues may also be modified. To estimate the number of tyrosine residues modified and their subsequent deacetylation, the method of Riordan and Vallee (1967 b)[41] was followed. The acetylated protein was mixed with an equal volume of 0. M hydroxylamine hydrochloride in distilled water (pH adjusted to 7.5 with 2 M NaOH) and the absorbance at at 278 nm was monitored continuously over a period of one hour. The initial absorbance was taken to be that for

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deacetylated OMDUK III. The number of O-acetylated tyrosine residues (N) was calculated from the equation:

$$N = (\Delta A_{278} \times M) / (1160 \times c)$$

Where ΔA_{278} is the difference in absorbance at 278 nm for the O- acetylated and deacetylated proteins, M is the molecular weight of the protein, and c is the concentration of the protein.

Succinylation of OMDUK III

The protein was succinylated according to the method of Klotz using succinic anhydride. Appropriate amounts of succinic anhydride were added to the protein solutions (in 0. 2 M sodium phosphate buffer, pH 7.5) The reaction was carried out at 4° C and the pH was maintained between 7.0 and 7.8 by adding suitable aliquots of 2 M NaOH. Reactions were continued until pH changes stopped (about one hour).

Guanidination of OMDUK III

A 0.5 M solution of O – methyl isourea was prepared in distilled water and its pH was adjusted to 10.5 with 2 M NaOH. Appropriate volumes of this solution were taken for 33%, 67% and 100% guanidination of the protein The pH was maintained at 10.5 during the reaction which was carried out for for 24 hours at room temperature (Kimmel, 1967)[42].

All modified preparations were dialyzed extensively against 0.06 M sodium phosphate buffer pH 7.0 and stored at - 4° C.

Quantification of modification

2, 4, 6 – trinitrobenzene sulfonic acid (TNBS) was used for the quantification of lysine residues modified. The method described by Habeeb (1966)[43] was followed for this purpose.

To 1 ml of protein solution (containing 0.05 to 0.5 mg of protein), 1 ml of 4% NaHCO3 and 1 ml of 0.1% solution of TNBS in distilled water were added. After an incubation of two hours ast 40 °C, the reaction was stopped using 1 ml of each of 10% SDS and 1N HCl (hydrochloric acid). Absorbance was read at at 335 nm against reagent blank prepared similarly but with 1ml distilled water instead of protein solution. The percent modification achieved at a particular molar excess nm vs. the amount of protein using the equation:

$$\% \text{ Modification} = 100 [1 - (m2 / m)]$$

Where m2 is the slope of the modified protein and m is that of the unmodified protein.

Characterization of the modified derivatives

Size and charge homogeneity of the unmodified and modified OMDUK III derivatives was checked by size exclusion chromatography and polyacrylamide gel electrophoresis under native conditions.

The proteins were characterized in terms of their Stokes' radii (using Sephadex G 50 column, 1.95 x 73.3 cm), relative mobilities upon native – PAGE, UV – absorption spectra and emission spectra.

The inhibitory activities of the unmodified and modified derivatives were studied against the serine proteinases chymotrypsin, subtilisin Carlsberg, and SGPB (*S.griseus protease* B), as described earlier.

Urea induced transition studies

The urea induced transition produced in the unmodified and modified derivatives of the third domain was studied as a function of inhibitory activity of the inhibitor molecule against chymotrypsin. Enzyme activity of chymotrypsin at increasing urea concentrations was obtained by incubation 0.05 mg of chymotrypsin and 0.05 mg of SGGPNA (succinyl-glycyl-glycyl-phenylalanyl-p-nitroanilide) at 36 ^{0} C for 20 minutes in presence of 8 µg of unmodified third domain at the different urea concentrations to determine the decrease in inhibitory activity occurring upon addition of inhibitor.

Eight µg of the different derivatives of duck ovomucoid third domain (unmodified and modified) were separately preincubated for 24 hours at room temperature in buffer containing increasing amounts of urea. To these solutions, enzyme and substrate were added followed by incubation at 36 ^{0} C for 20 mins as per the usual protocol. Results were interpreted in terms of enzyme activity and decrease in percent inhibition at increasing urea concentrations.

RESULTS

Isolation and characterization of third domain of duck ovomucoid

Duck ovomucoid third domain was isolated by treating pure ovomucoid with thermolysin and fractionating the reaction mixture on Sephacryl S-I00 HR col-

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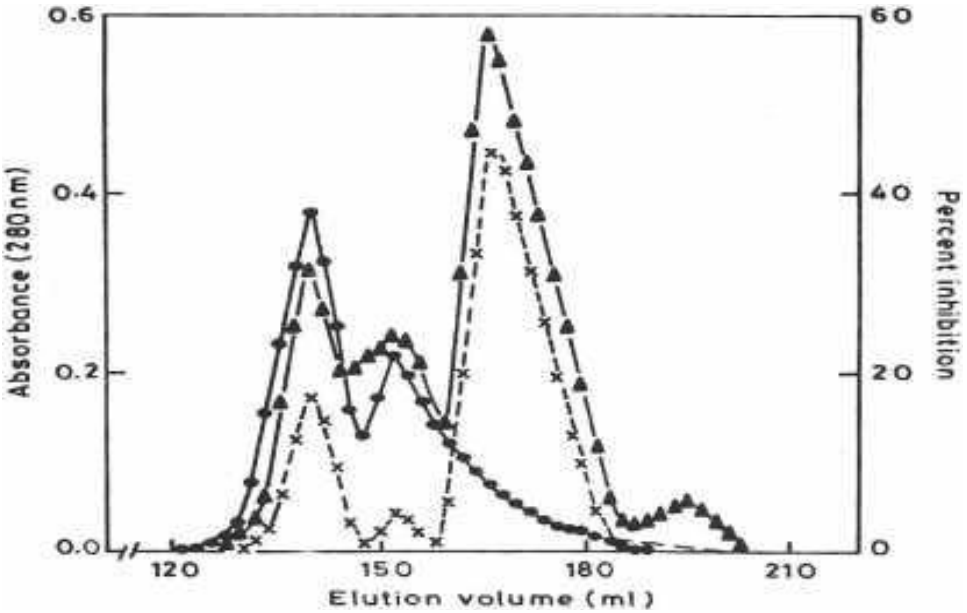

Figure 1

Elution profile of reaction mixture of ovomucoid and thermolysin on Sephacryl S-100 HR column. Fractions of 2 ml were collected and monitored at 280nm (●—●). From each fraction, aliquots of 0.01 ml were taken and separately monitored for antitryptic (△—△) and antichymotryptic (×- - -×) activities which were interpreted in terms of % inhibition.

TABLE 1

Characterization of ovomucoid and different peak proteins (obtained by passing reaction mixture of ovomucoid with thermolysin on Sephacryl S-100 HR column) Only peak fractions were taken

Property	Ovomucoid	Peak I	Peak IIa	Peak IIb	Peak III
% Carbohydrate content	22.20	34.05	20.01	26.80	17.73
Specific inhibition					
--Trypsin	39.76	48.54	5.03	60.04	1.09
--Chymotrypsin	20.14	15.12	2.00	2.25	57.06

umn. The elution profile showed three distinct peaks (Figure 1) Fractions under each peak was monitored for antitryptic and antichymotryptic activities. Peak III showed the highest amount of antichymotryptic activity. (TABLE 1).

Chemical modification of third domain of duck ovomucoid

In order to find out the percent modification obtained by using different molar excesses of the modifying reagent, duck ovomucoid third domain was subjected to chemical modification with varying concentrations of acetic anhydride. The extent of modification was determined by trinitrobenzene sulfonic acid (TNBS) as described in Materials and Methods section. The linear plots between the amount of protein and absorbance at 335 nm are shown in Figure 2(a). The extent of modification was determined from the slopes of these plots. Figure 2(b) shows a plot of percent modification versus molar excess of acetic anhydride. the presence of two types of residues can be seen from this plot: fast reacting and slow reacting. After about 50 molar excess of the modifying reagent at which about 35% modi-

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fication is achieved, the increase in the percent modification with increase in the concentration of the modifying reagent is small, suggesting that presumably all the exposed lysine residues have been modified at this reagent concentration. Since OMDUK III contains only three lysine residues, it was treated with 50, 500. 1000 molar excesses of the modifying reagents (acetic anhydride, o-methyl isourea and succinic anhydride) to obtain the modification of one, two or all three lysine residues respectively.

Estimation of percent modification of OMDUK III by TNBS method. Plots of absorbance at 335 nm vs. protein concentrations for the calculation of the slope values for the different acetylated derivatives.

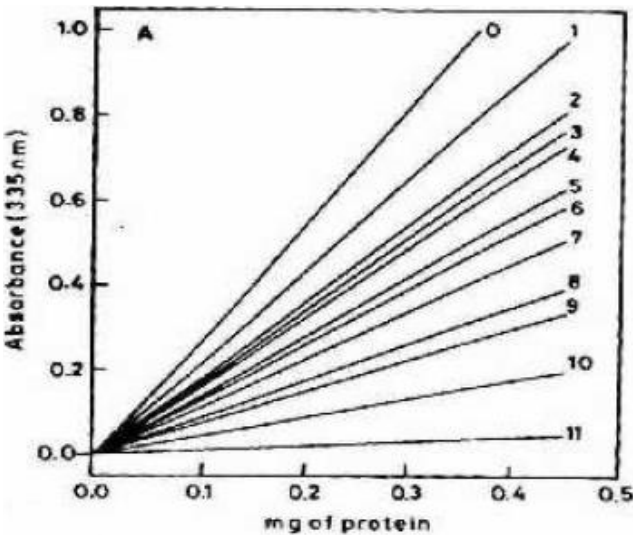

Figure 2 (a)

Plot of percent modification achieved at increasing molar access of acetic anhydride

Both guanidination and succinylation performed under the conditions used in this study have been reported to be highly specific for the amino groups of the proteins. However, acetylation is not that specific and may result in the modification of tyrosine residues also. The acetylated derivative was therefore treated with hydroxylamine hydrochloride (as in Materials & Methods) and two of the three lysine residues present in the third domain were found to be acetylated. Consequently, this preparation was first treated with hydroxylamine hydrochloride for one hour to remove the acetyl groups from the tyrosine residues and then extensively dialyzed against 0.06 M sodium phosphate buffer pH 7.0

The purity of the modified derivatives of OMDUK III was checked by gel filtration on Sephadex G-50 column and polyacrylamide gel electrophoresis under native conditions. Figure 3 (a,b,c) shows the elution pro-

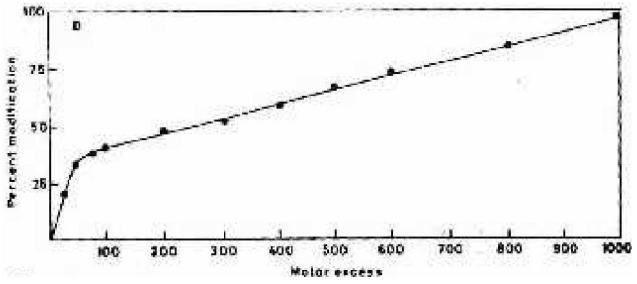

Figure 2 (b)

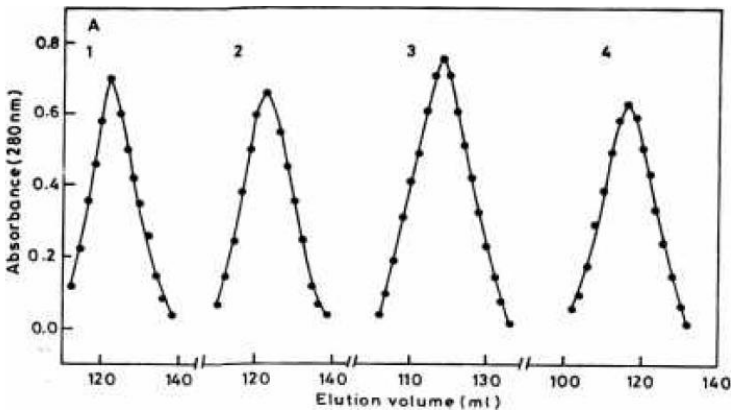

Figure 3 (a)

Elution profiles of unmodified and modified derivatives of OMDUK III on Sephadex G-50 column (details same as in Figures 22 and 23)

a. Unmodified (1) and 33 % (2), 67 % (3), and 100 % (4) acetylated derivatives of OMDUK III.

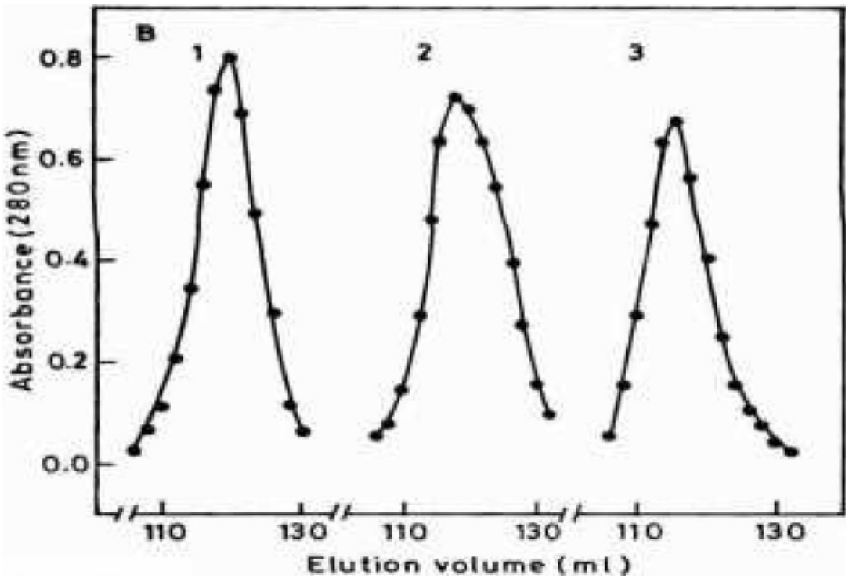

Figure 3 (b)

33 % (1), 67% (2), and 100 % (3) Guanidinated derivatives of OMDUK III

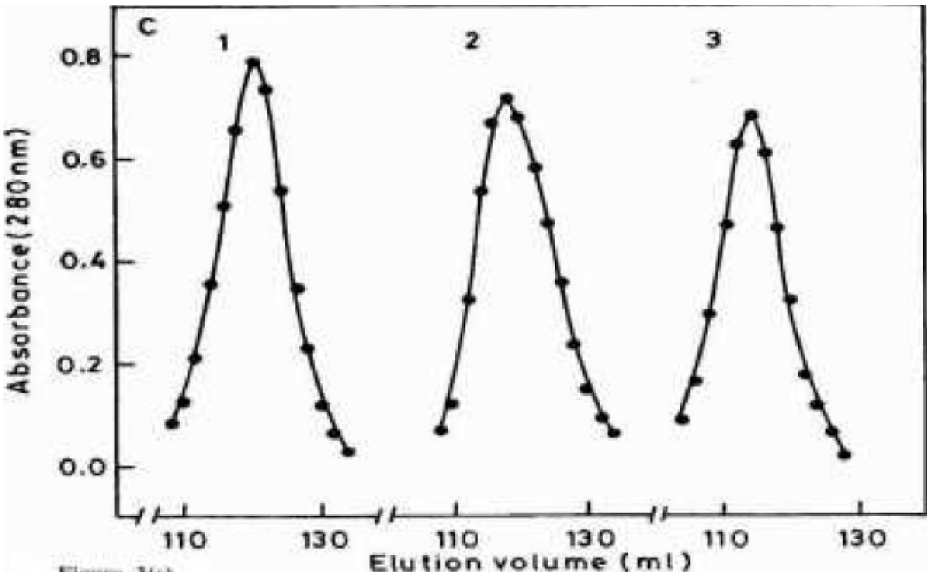

Figure 3 (c)

33 % (1), 67 % (2), and 100 % (3) Succinylated derivatives of OMDUK III.

files of the modified preparations. A single sharp peak in each case was indicative of size homogeneity of these preparations All the modified preparations gave a single band on polycrylamide gel electrophoresis indicating charge homogeneity of the modified derivatives.

CHARACTERIZATION

The different chemically modified preparations were characterized in terms of their Stokes' radii, relative mobility on 12% gel under native conditions, wavelength of maximum absorbance, emission wavelength, and inhibitory activity against different serine proteinases.

(a) Determination of Stokes' radius: A calibrated Sephadex G-5- column, equilibrated with 0.06 M sodium phosphate buffer, pH 7.0, was used for the determination of the Stokes' radii of the modified derivatives (TABLE 2). The chromatographic profiles of the different modified derivatives are shown in Figure 3 and Figure 4 and TABLE 3 show the treatment of the gel filtration data according to Laurent and Killander (1964)[44] and Ackers

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(1967)[45].

(b) Native polycrylamide gel electrophoresis: The R_m values of unmodified and modified OMDUK III preparations, obtained on 12.5% polycrylamide gel under native conditions are listed in TABLE 4.

(c) Spectral properties: ultraviolet absorption spectra and fluorescence emission spectra of the unmodified and modified OMDUK III derivatives were taken in 0.06 M sodium phosphate buffer pH 7.0 (Since the spectra were similar in shape to the absorption and emission spectra for unmodified duck ovomucoid third domain, they have been omitted for brevity). The wavelength of maximum absorbance of the derivatives and the emission maxima are listed in TABLE 4.

(d) Specific inhibition: Specific inhibition of the serine proteinases chymotrypsin, subtilisin Carlsberg, and SGPB by unmodified and modified derivatives of OMDUK III was determined by taking 0.05 mg enzyme, 8μg inhibitor and 0.05 mg SGGPNA (substrate for chymotrypsin) or 7.5 mg casein (substrate for subtilisin Carlsberg and SGPB)

Analysis of gel filtration data according to the methods of Laurent and Killander[44] and Ackers[45] for the estimation of Stokes' radii of the unmodified and modified derivatives of OMDUK III. (Figure 4a & 4b)

Urea-induced transition studies

urea induced conformational transition was studied between 0 to 9 M urea concentrations by following the decrease in specific inhibition of chymotrypsin. The loss in the activity of chymotrypsin occurring as a result of urea-induced conformational alterations was monitored by measuring the enzyme activity between 0 to 9 M urea concentrations (Figure 5). To find out if the addition of unmodified inhibitor affects the enzyme activity, the enzyme activity was also measured in the presence of 8 μg of OMDUK III at the different urea concentrations. (Figure 5).

For studying the effects of urea on the inhibitory activity of unmodified and modified OMDUK III, 8 μg each of the different derivatives were separately preincubated for 24 hours at room temperature in 1.5 ml of working buffer solution containing varying amounts of urea. These were subsequently added to the enzymes and the usual protocol was followed. To determine the enzyme activity (Figure 6). The percent decrease in specific inhibition was plotted against urea concentration to check the increase or decrease in stability occurring upon chemical modification (Figure 7). TABLE 5 lists the difference in enzyme activity between unmodified and modified OMDUK III derivatives in the absence of urea, and the difference in percent decrease in inhibition for each derivative at 0 and 9 M urea concentrations.

Enzyme activity in the presence of unmodified and modified OMDUK III derivatives preincubated in in-

TABLE 2

Treatment of gel filtration data according to the methods of Laurent and Killander (1964) and Ackers (1967) for the estimation of Stokes' radius of duck ovomucoid third domain. Sephadex G-50 column (1.95 x 73.3 cm^2) equilibrated with 0.06 M sodium phosphate buffer, pH 7.0, and operated at a flow rate of 30 ml/h was used.

Protein	Stokes' radius (nm)	V_e (ml)	K_{av}	$(-\log K_{av})^{1/2}$	K_d	$erfc^{-1} K_d$
Carbonic anhydrase	2.57	93.0	0.035	1.209	0.042	1.445
Trypsinogen	2.34	106.5	0.138	0.927	0.166	0.980
Ribonuclease	1.92	119.5	0.238	0.790	0.286	0.748
Cytochrome	1.64	125.0	0.280	0.744	0.336	0.679
OMDUK III	---	122.0	0.257	0.768	0.309	0.717

creasing urea concentrations for 24 hours at room temperature.(Figure 6(a), (b) and (c))

DISCUSSIONS

Chemical modification of OMDUK III

In order to find if the reactivity of the three lysine residues of OMDUK III is different, the peptide was subjected to chemical modification with increasing acetic anhydride concentrations (Figure 2). Figure 2 b clearly shows the presence of two types of lysine residues, fast reacting and slow reacting. It is apparent from the Figure that of the three lysine residues present in the OMDUK III fragment, one is exposed while the other two are relatively inaccessible. This is also supported by the crystal structures and ribbon drawings of the

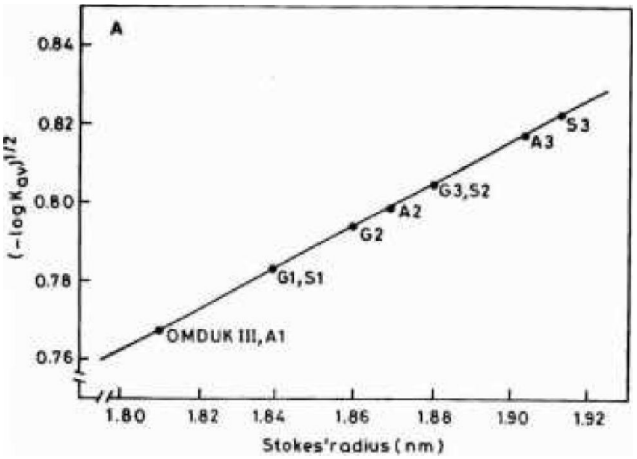

Figure 4 (a)

Gel filtration data analysis according to the method of Laurent and Killander (1964).

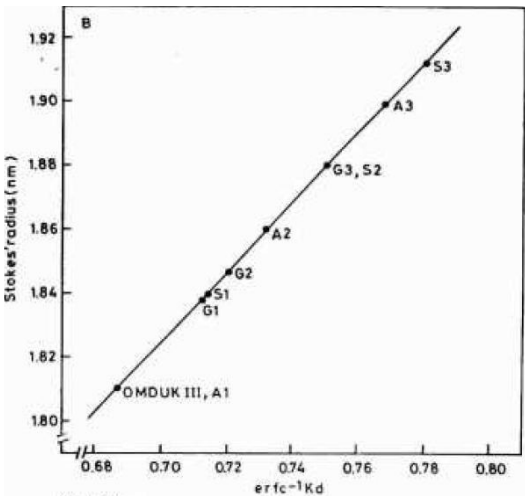

Figure 4 (b)

Gel filtration data analysis according to the method of Ackers (1967)

TABLE 3

Treatment of gel filtration data obtained on Sephadex G-50 column (1.95x73.3 cm^2) according to the methods of Laurent and Killander (1964) and Ackers (1967) for estimation of Stokes' radii of OMDUK III derivatives. Details same as in Table2

Protein	V_e	K_{av}	$(-\log K_{av})^{1/2}$	K_d	$erfc^{-1}K_d$	Mean Stokes' radius (nm)
OMDUK III	122	0.257	0.768	0.309	0.717	1.81
A1OMDUK III	122	0.257	0.768	0.309	0.717	1.81
A2OMDUK III	118	0.226	0.803	0.272	0.765	1.88
A3OMDUK III	116	0.211	0.822	0.253	0.791	1.92
G1OMDUK III	121	0.254	0.775	0.296	0.722	1.84
G2OMDUK III	120	0.242	0.785	0.290	0.741	1.86
G3OMDUK III	117.5	0.222	0.808	0.267	0.772	1.89
S1OMDUK III	121	0.254	0.775	0.296	0.772	1.84
S2OMDUK III	117.5	0.222	0.808	0.267	0.772	1.89
S3OMDUK III	115	0.203	0.832	0.249	0.796	1.93

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TABLE 4

Summary of characterization parameters of unmodified and modified derivatives of duck ovomucoid third domain

Derivative	R_m	λ_{max}	Em_{max}
OMDUK III	0.088	279.17	303
A1 OMDUK III	0.147	279.17	303
A2 OMDUK III	0.225	279.17	304
A3 OMDUK III	0.314	280.30	305
G1 OMDUK III	0.078	279.17	303
G2 OMDUK III	0.069	279.75	304
G3 OMDUK III	0.039	280.30	305
S1 OMDUK III	0.216	279.75	304
S2 OMDUK III	0.353	280.30	305
S3 OMDUK III	0.392	280.75	305

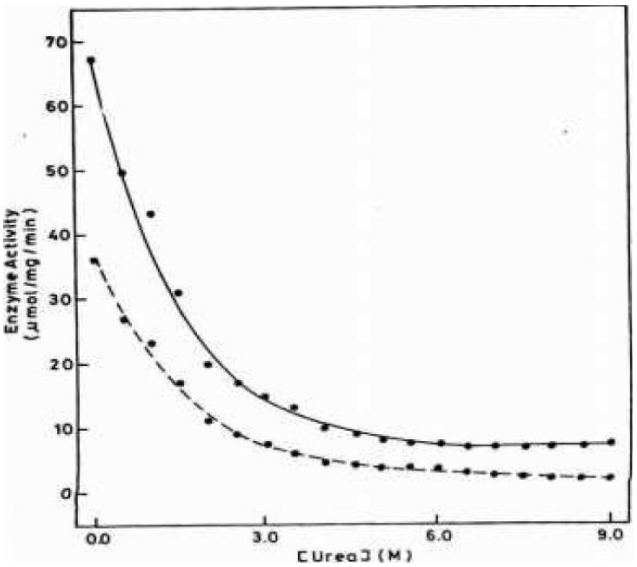

Figure 5

Activity of chymotrypsin in the absence (●—●) and presence (●-●) of OMDUK III at increasing urea concentrations.

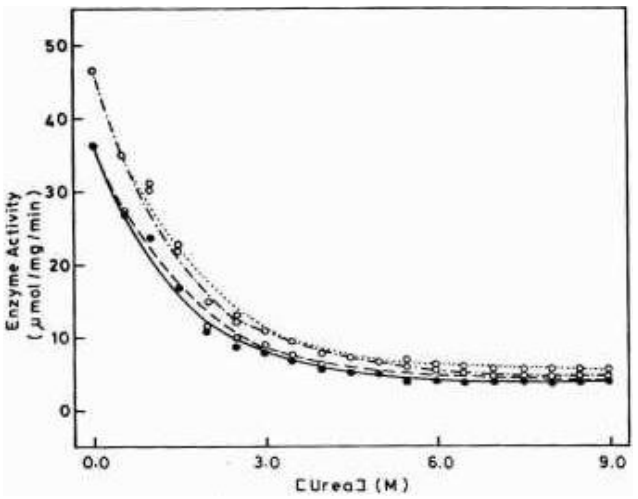

Figure 6 (a)

Unmodified (●—●) and 33 % (○- - -○), 67 % (○ ···○), and 100 % (○····○) acetylated OMDUK III.

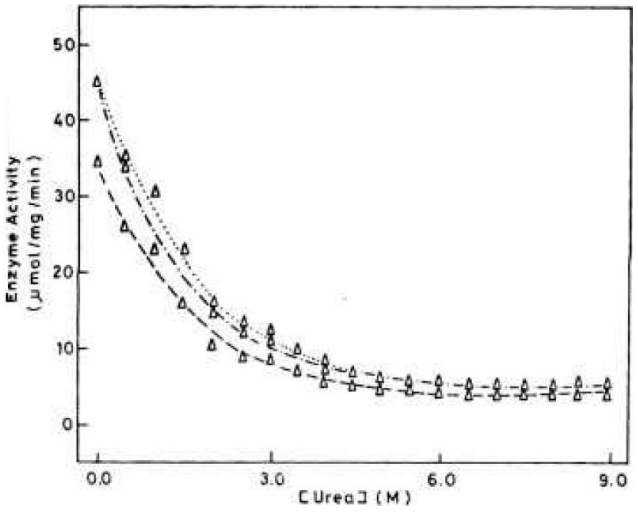

Figure 6 (b)

33 % (Δ—Δ), 67 % (Δ- -Δ), and 100 % (Δ ···Δ) guanidinated OMDUK III

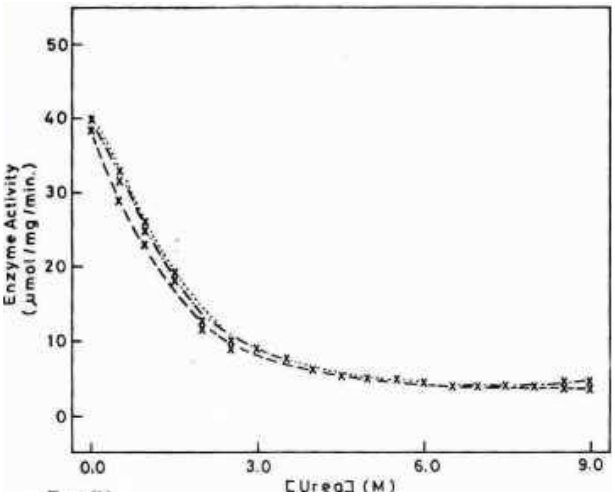

Figure 6 (c)

33 % (x- - -x), 67 % (x—x), and 100 % (x ····x) succinylated OMDUK III

third domain fragment[45]. Wright and Scarsdale[46] have also shown that the reactive loop is protruded outside the nucleus of the molecule. In view of this, it is probable that the lysine residue present near the reactive site peptide bond is in an exposed position in the reactive site loop. The other two lysine residues are present in the nucleus of the molecule, relatively shielded from the environment by the neighboring amino acid residues. This experiment was a prerequisite for determining the molar excesses of modifying reagent required for obtaining 33%,67% and 100% modified derivatives

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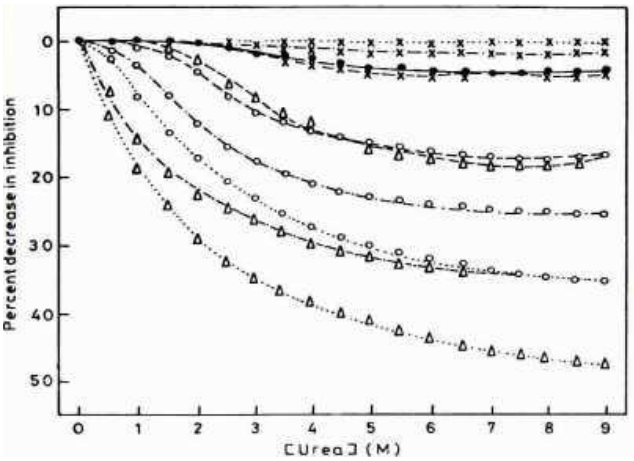

Figure 7

Per cent decrease in inhibitory activity against chymotrypsin at increasing urea concentrations upon modification of OMDUK III

The different derivatives were : unmodified OMDUK III (●—●), A1 (O---O), A2 (O--O), A3 (O···O), G1 (x---x), G2 (x--x), G3 (x···x), S1 (Δ--Δ), S2 (Δ--Δ), and S3 (Δ--Δ).

TABLE 5 (a)

Percent decrease in the enzyme inhibitory activity obtained upon the chemical modification of OMDUK III

Modified derivative	Percent decrease in inhibition of		
	Chymotrypsin	Subtilisin Carlsberg	SGPB
A1	4.2	4.0	3.9
A2	10.2	10.0	9.9
A3	14.2	13.9	13.8
G1	6.9	7.0	6.9
G2	8.7	8.5	8.4
G3	9.7	9.6	9.3
S1	- 6.3	7.0	6.9
S2	11.2	11.0	12.0
S3	15.2	15.1	15.8

(acetylated, succinylated and guanidinated) of the third domain.

Size and charge homogeneity of the modified derivatives were confirmed by size exclusion chromatography (Figure 3) and polycrylamide gel electrophoresis under native conditions. All the derivatives gave a single peak on gel filtration and only one band upon electrophoresis, excluding the possibility of any major size or charge heterogeneity in the chemically modified preparations.

TABLE 5 (b)

Percent decrease in specific inhibition occurring between 0 and 9 M urea concentration for the unmodified modified derivatives of OMDUK III

Derivative	Specific inhibition at		% decrease
	0.0 M	9.0M	
OMDUK III	57.21	54.92	4.0
A1	54.82	47.97	12.5
A2	51.35	38.25	25.5
A3	49.07	31.89	35.0
G1	53.29	46.90	12.0
G2	52.25	51.47	1.5
G3	51.63	51.63	0.0
S1	60.79	50.46	17.0
S2	50.78	33.1	34.8
S3	48.50	25.46	47.5

Stokes' radii of the modified derivatives were determined from chromatographic data. The largest increase in stokes radius was seen in 100% succinylated OMDUK III (from 1.83 nm to 1.93 nm) This is understandable since succinylation of each lysine residues increases the net negative charge on the protein by two units[47]. On the other hand, 33% acetylated derivative had the same value of stokes radius as unmodified OMDUK III. All the other modified derivatives had the stoke's radius ranging between 1.83 nm and 1.93 nm.

The electrophoretic mobility of the different preparations varied with the extent of negative charge imposed upon modification. Thus, the 100% succinylated derivative had maximum mobility while the 100% guanidinated had the least mobility. The guanidinated derivatives had relative nobilities approaching that for the unmodified OMDUK III since all these derivatives had identical net charges.

Slight red shifts in the λ_{max} of the ultraviolet spectra (maximum shift of 1.58 nm occurring upon succinylation of OMDUK III)as well as in the emission maxima of fluorescence spectra (maximum shift of 2.0 nm occurring upon 100% modification) are a consequence of conformational changes that accompany chemical modification.

Although the changes occurring upon modification for an individual parameter (Stoke's radius/ λ_{max}/ emission maximum) are too small to be of importance, these results when considered conjointly, support each other and suggest that a conformational change occurs upon

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67% and 100% modification. The maximum conformational change occurs upon 100% succinylation. The index of conformational change was:

S3 > A3 > G3 = S2 > A2 >G2 > S1 = G1 >A1 = OMDUK III

Specific inhibition of the serine proteinases chymotrypsin, subtilisin Carlsberg, and SGPB by the unmodified and modified derivatives showed interesting results Loss in inhibitory activity occurred upon chemical modification in all cases except the inhibition of chymotrypsin by 33% succinylated OMDUK III. Further, the percent loss in activity occurring upon acertain degree of modification was almost equal for the three enzymes, except the inhibition of chymotrypsin by 33% succinylated derivative. A comparison of the inhibitory activities of the 33% acetylated and guanidinated derivatives showeed that the percent loss in inhibitory activity was almost 4% for the acetylated derivative and 7 % for the guanidinated derivative. It is important to note that in both these preparations only one lysine residue, which is present in exposed position near the reactive site peptide bond, was modified.. Taken together, these results suggest that the positive charge of lysine residue at this position is not absolutely necessary for the inhibitory activity of OMDUK III. The small decrease in inhibitory activity occurring upon neutralization of this charge by acetylation is probably due to the steric hindrance created by the acetyl group.

A further loss in inhibitory activity occurred upon 67 and 100 % acetylation and guanidination. However, in these preparations, the percent decrease in inhibitory activity was greater upon acetylation (10 and 14% for 67 and 100% modified derivatives respectively). In these preparations, the two remaining lysine residues modified are far away from the reactive site and should not affect the approach of the enzyme in any way. Probably, the modifications of these lysine residues affected the conformation of the inhibitor molecule, thereby reducing its inhibitory activity. This result is also supported by the alterations in Stokes' radii $\lambda_{max,}$ and emission maxima for these derivatives. A slightly higher decrease (11 and 15% in 67% and 100% succinylated derivatives, respectively) can be explained in terms of maximum conformational alteration in these preparations. In contrast, the 33% succinylated derivative showed a 6.5% increased inhibitory activity against chymotrypsin. Inhibitory activities against subtilisin Carlsberg and SGPB decreased by 7% upon 33% succinylation of OMDUK III (as also in case of 33% guanidination). A study of the structure of the chymotrypsin active showed the presence of a positive charge contributed by histidine-57 near the active site. Favorable interactions between this residue of the enzyme with the negative charge introduced by succinylation of OMDUK III are probably responsible for the increased inhibitory activity of the 33% succinylated preparations.

Urea - induced transition

Urea induced decrease in the chymotrypsin inhibitory activity of the OMDUK III was used as a parameter for studying the stability of this fragment. In the presence of urea, the activity of chymotrypsin was found to decrease with increasing urea concentrations. Addition of a fixed amount of unmodified inhibitor produced a further decrease in the enzyme activity. These results suggested that the effect of urea on the inhibitor molecule could be monitored as afunction of enzyme activity.

Unmodified and modified OMDUK III derivatives, preincubated for 24 hours at varying urea concentrations, were used to inhibit the activity of chymotrypsin. The percent decrease in the inhibitory activities of these derivatives showed that the maximum decrease in inhibition occurred upon 100% succinylation and the least upon 100% guanidination. If the percent decrease in inhibitory activity is assumed to be directly related to the stability of the peptide in urea, it would appear that stability decreases in the following order:

G3 > G2 > OMDUK III > G1 = A1 > S1 > A2 > A3 = S2 > S3

The results show the importance of positively charged lysine residues in the stability of the third domain nucleus. Also, since the introduction of a bulky, positively charged modifier is increasing the stability of the molecule (as in 67% and 100% guanidinated preparation) it would appear that the interior of the third domain is not sufficiently compact in the unmodified form.

Chemical modification studies of the ovomucoid third domain lead to the following conclusions:

1. Acetylation of duck ovomucoid third domain with increasing molar excesses of the modifying reagent showed that of the three lysine residues present in the third domain, the one near the reactive site is in an exposed position while the other two are relatively buried.

2. 33% acetylated and succinylated derivatives of the third domain showed a very slight decrease in inhibitory activity against the serine proteinases chymotrypsin,subtilisin Carlsberg and SGPB indicating that the positive charge of the exposed lysine residue is not crucial for the inhibitory activity of the domain. However, the presence of modifying group interferes with the approach of the enzyme in the enzyme – inhibitor complex formation.
3. Positive charges of the two buried lysine residues are not directly required for the inhibitory activity. The conformational changes occurring upon modification alter the structure of the reactive site sufficiently to produce loss in inhibitory activity.
4. Although a conformational alteration is produced upon guanidination of the third domain which reduces the inhibitory activity of the molecule, the stability of the altered conformation is much higher than that of the original.

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