

Protein proteinase inhibitors from avian egg whites

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Received 6 March 1996; received after revision 17 June 1996; accepted 10 July 1996

Abstract. Avian egg whites are a rich source of protein inhibitors of proteinases belonging to all four mechanistic classes. Ovomucoid and ovomucoid are multidomain Kazal-type inhibitors with each domain containing an actual or putative reactive site for a serine proteinase. Cystatin is a cysteine proteinase inhibitor, while ovostatin inhibits proteinases of all four mechanistic classes. In this review we have summarized the general features, isolation, inhibitory mechanism and evolutionary aspects of these inhibitors.

Key words. Proteinase inhibitors; ovomucoid; ovomucoid; ovostatin; cystatin; ovalbumin.

Introduction

The ability of chicken egg white to inhibit trypsin was recognized in the early part of the twentieth century [1] and was attributed to ovomucoid [2]. At present, three different proteins of the avian egg white are known to possess trypsin inhibitory activity: ovomucoid, ovomucoid and ovostatin [2–4]. Besides these, one other protein proteinase inhibitor is also present – cystatin, which inhibits cysteine proteinases [5]. Currently, the comparative properties of protein proteinase inhibitors of closely related species are receiving increasing attention as an approach to understanding their structure-function relationship. Avian ovomucoid, and to some extent ovomucoid, are excellent candidates for achieving the above goal, since highly homologous proteins with identical/similar properties can be obtained from different species. This review is not a complete summary of the literature on these inhibitors but is an attempt to present some of the relevant information in a coherent form.

Ovomucoid

Ovomucoid accounts for 10% of the protein produced by the tubular gland cells of the oviduct in laying birds [6]. The molecular weight ranges between 27 and 35 kD [2, 7, 8]. About 20–25% carbohydrate content has been reported [9]. The variable carbohydrate content observed in ovomucoid preparations obtained from a single species is probably due to incomplete specificity of the biosynthetic enzymes [10].

Isolation of ovomucoid

The isolation procedure originally described by Lineweaver and Murray [2] involves TCA-acetone

precipitation followed by precipitation with acetone, and gives a recovery of 40–50% with 80% purity. A modification of this procedure, involving gel filtration on Bio-Gel P10 column and CM-Sepharose chromatography, yields a considerably purer preparation and is currently used [11]. Recently, affinity chromatography of lysozyme-, avidin- and ovotransferrin-free egg white protein solution (obtained by CM-Cellulose chromatography) on Blue Sepharose CL 6B or chelating Sepharose 6B columns has been used for purification of ovomucoid preparation, with 77.9% recovery [12].

Primary structure and physicochemical properties

Kato and his group [13] gave the primary structure of chicken ovomucoid, amended later at three places and confirmed by cDNA studies [14]. A 24-amino acid residue signal sequence is removed during the synthesis of the protein. The single polypeptide chain is organized into three homologous Kazal-type domains, all of which have actual or putative reactive sites. The molecule may accordingly be single, double or triple headed. For example, chicken, ostrich and cassowary ovomucoids are single headed for trypsin, while golden pheasant ovomucoid is single headed for chymotrypsin. Turkey, penguin, tinamou, rhea and guinea ovomucoids are double headed with one reactive site for trypsin and one for chymotrypsin, subtilisin and elastase. Triple-headed ovomucoids with two reactive sites for trypsin and one for chymotrypsin, subtilisin and elastase are from duck and emu [11]. The Asn-X-Thr/Ser sequence is repeated six times in the entire sequence of ovomucoid and is a possible glycosylation site. Of these, the Asn¹⁵⁸-Lys-Thr sequence, lying in the third domain, is not glycosylated and has also been found unglycosylated in other avian ovomucoids [15]. The glycosylation sites at Asn¹⁰-Ala-Thr and Asn⁷⁵-Thr-Thr are at homologous positions in the first and second domains [11].

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Table 1. Physicochemical properties of proteinase inhibitors from avian egg whites.

Property	Value			
	avian ovomucoids	chicken ovoinhibitor	chicken ovostatin	chicken cystatin
Molecular weight				
– Sedimentation equilibrium	27,000 [7]	48,600	700,000– 780,000 [4, 65] dimer 300,000 [4] monomer 175,000 [4]	-
– Amino acid analysis	27,300 [7]	49,000 [53]	-	13,000 [72, 74]
Carbohydrate content	20–25% [9]	10%	0.28%	-
Isoionic point	4.3–4.5 [10]	-	4.9 [65]	Form I 6.5 [71] Form II 5.6 [71]
Sedimentation constant ($S_{20,w}$, $\times 10^{13}$)	-	3.3	16.97 [65]	-
Diffusion coefficient ($D_{20} \times 10^7$, cm^2/S)	6.01–8.28 [7]	-	2.43 [65]	-
Intrinsic viscosity	4.7–5.46	-	6.51 [65]	-
Partial specific volume (v , ml/g)	0.697 [7]	-	0.744 [65]	-
$E_{1\text{ cm}}^{1\%}$	4.13–6.1 [7]	7.4	-	8.7 [11]
Helical content	26% [8]	-	-	20%
β structure	46% [18]	-	-	80%
Random coil	18% [18]	-	-	-

There is no corresponding sequence in the third domain. Two other glycosylation sites at Asn⁵³-Ile-Ser and Asn⁶⁹-Cys-Ser are also present in domains I and II, respectively; the latter is unusual and has thus far been seen only in chicken and Ceylon jungle fowl ovomucoids. The carbohydrate moieties of ovomucoid of a single species differ in composition and length. For example, duck ovomucoid bears more than 16 different asparagine-linked oligosaccharides [16]. Ovomucoids from most species have five glycosylation sites.

Table 1 lists some of the physicochemical properties of the protein. Ovomucoid is a fairly asymmetric molecule, as is evident from its hydrodynamic properties (see table 1). Tryptophan is absent in ovomucoid. About half of the tyrosyl residues of the protein are exposed under native conditions, as shown by solvent perturbation difference spectroscopy [17]. Ovomucoid is rich in β -structure (46%) compared with α -structure (26%), as studied by circular dichroism [18]. The first five third-domain sequences reported by Laskowski's group (chicken, turkey, ring-necked pheasant, Japanese quail and chachalaca) have five different residues at the reactive site position, P₁ (Ala, Leu, Met, Lys and Gln, respectively). This extreme hypervariability of reactive site residue was considered unusual and could be present at the race, species or genus level. It was also uncertain if the hypervariability is limited to P₁ residue or encompasses all residues involved in the enzyme-inhibitor contact. Subsequently, Laskowski's group sequenced the third domain from different avian species [15, 19, 20], and this set of sequences was found to

provide an excellent opportunity for reactivity studies as well as for studies of molecular properties such as inhibition specificity, immunological cross-reactivity, stability, nuclear magnetic resonance (NMR) and optical spectra, chromatographic behaviour, and the evolutionary aspects of these proteins. NMR studies of different ovomucoid third domains suggest that they behave as a rigid, native, globular protein with high melting temperatures [21]. Denaturation studies of ovomucoid and its isolated domains suggest that these domains fold independently into a compact shape with a hydrophobic core [17, 21–24]. The third domains of silver pheasant, Japanese quail and turkey ovomucoids (virgin and modified) have been isomorphically crystallized in the monoclinic space group C2 with cell dimensions of $a = 4.429$ nm, $b = 2.115$ nm, $c = 4.405$ nm and $\beta = 107^\circ$ [25–27]. The asymmetric unit was found to have one molecule, corresponding to an extremely low volume per molecular mass of 0.0017 nm³/D. The spatial atomic structures of turkey ovomucoid third domain with *Streptomyces griseus* proteinase B, bovine chymotrypsin and human elastase have been determined by X-ray diffraction [21, 28]. The results show that 8 of the 11 enzyme-inhibitor contact positions are strongly hypervariable. The solution structure of turkey ovomucoid third domain (virgin and reactive site hydrolysed) has been determined by NMR spectroscopy [29–32]. About 70 unique sequences have been reported from the third-domain sequences of 150 avian species, of which 40 are related to their nearest neighbour by one amino acid substitution [15, 19, 20]. The following generalizations have been made from these studies:

1. Residues which determine the folding (proline, glycine and disulfide-bridged cysteine) are highly conserved.
 2. The 8 positions which have the largest number of alternatives are amongst the 11 that are in contact with the enzyme in the enzyme-inhibitor complex. This hypervariability of reactive-site residues in members of the serpin family is a frequently encountered phenomenon rather than a rarity [33–35]. The hypervariability is not limited to the third domain but may occur in the first and second domains. Ardelt and Laskowski [36] have shown that eight different proteinases, including chymotrypsin, subtilisin, elastase and human furin [37], are inhibited by the third domain of turkey ovomucoid at the same reactive site, indicating that the contact sites on the inhibitor are the same for all enzymes. Any change in the contact residues affects the K_{assoc} values for enzyme-inhibitor complex formation.

The biological function of proteinase inhibitory activity of ovomucoid is not known. It could be bacteriocidal with the rapid evolution in the reactive-site residues occurring in response to differences in bacterial flora likely to infect the egg whites of a given species, or it could be directed against the bird's own proteinases (probably those involved in some of the final events of embryonic tissue differentiation) [15]. In any case, ovomucoids and their isolated domains are powerful inhibitors of serine proteinases, and the homology of the individual domains to the pancreatic secretory inhibitor (whose physiological function is clearly the inhibition of trypsin) is very strong, indicating that the proteinase inhibitory activity of ovomucoid is not accidental.

The reactive sites

The reactive site is a single special peptide bond in each inhibitor molecule or domain which serves the cognate enzyme as a high-affinity substrate. A six- or seven-amino acid residue sequence is involved in the complex formation between inhibitor and enzyme.

The hydrolysis of the reactive-site peptide bond converts the single-chain protein to a double-chain protein with the two fragments held together by disulfide bridges. The first and second domains usually have Lys or Arg as P_1 residues and inhibit trypsin-like enzymes. The third domain, however, has been found to have Lys as P_1 residue only in a few sequences. Arginine as P_1 residue has not been found (fig. 1).

A comparison of avian ovomucoid third domains shows that Leu or Met are usually present at position 18 and that they inhibit chymotrypsin, subtilisin, elastase and similar enzymes. Bobwhite quail ovomucoid third domain has Ser¹⁸-Glu¹⁹ at the putative reactive site. It is a strong inhibitor of subtilisin and elastase but does not inhibit trypsin or chymotrypsin. Japanese quail ovomucoid third domain has Lys at position 18 and inhibits trypsin. Apparently, the P'_1 site can tolerate a broad

range of residues. Reactive sites with Pro in the P'_1 position are not active as inhibitors. Silver pheasant ovomucoid with Cys-Asn-Lys-Ala as the reactive-site sequence inhibits trypsin, while highly homologous golden pheasant ovomucoid with Cys-Asn-Lys-Pro does not [33]. Covalent hybrid formation technique has recently been used to augment the set of known third domain sequences for the study of inhibitory properties [38]. In this technique, the reactive-site peptide bond is specifically hydrolyzed and the disulfide bridges reduced. The NH_2 -terminal fragment of one species is mixed with the COOH -terminal fragment of another, and the disulfide bridges are allowed to reform. Subsequently, the reactive-site peptide bond is enzymatically resynthesized. Synthetic N-terminal peptide (residues 6–18) and some natural C-terminal peptide (residues 19–56) have been used to produce new variants [39]. Structure-function studies on semisynthetic variants of ovomucoid third domain have been performed [40]. On the basis of available data, Laskowski's group [41] compared the relative effect of various residues at the contact positions for two enzymes to design the strongest possible inhibitor for a given enzyme, and predicted its K_{assoc} . Such strong inhibitors have not been found in nature.

A sequence to reactivity algorithm (a stepwise program for the prediction of the effect of a particular amino acid residue at a specific position in the inhibitor on the inhibitory activity) was developed by Laskowski's group [42–44]. Residues which are sequentially quite distant from the reactive site may exert great influence on the enzyme-inhibitor reaction provided that they make contact with the enzyme in the enzyme-inhibitor complex.

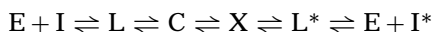
A rapid computational method has been developed to predict the relative binding strengths of small molecular weight ligands for the serine proteinase trypsin [45]. Binding-strength calculations were done directly for 18 different compounds, including non-binding controls, and were predicted successfully. The correctness of theoretical calculations was demonstrated with both kinetic measurements and X-ray determination of six enzyme-inhibitor complexes.

	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4
Japanese quail	Cys	Pro	Lys	Asp	Tyr	Arg	Pro
Ostrich	Cys	Pro	Leu	Asp	Tyr	Met	Pro
Emu	Cys	Ser	Leu	Glu	Tyr	Met	Pro
Pheasants (5 species)	Cys	Thr	Met	Glu	Tyr	Arg	Pro
Duck	Cys	Thr	Met	Glu	Tyr	Met	Pro
Turkey, Chukar, California, Gambel's and scaled quails	Cys	Thr	Leu	Glu	Thr	Arg	Pro
Bobwhite quail	Cys	Met	Ser	Glu	Tyr	Arg	Pro
Goose	Cys	Thr	Val	Glu	Tyr	Met	Pro
Chicken	Cys	Thr	Ala	Glu	Asp	Arg	Pro
Chacalaca	Cys	Leu	Gln	Glu	Gln	Lys	Pro

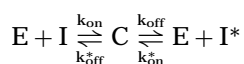
Figure 1. Reactive-site sequences of ovomucoid third domains of some avian species.

Inhibitory mechanism

Ovomucoid follows the same inhibition mechanism as most other small protein inhibitors of serine proteinases. The scheme is shown below:



where E is the enzyme, I is the virgin inhibitor (reactive-site peptide bond intact), L is the loose, noncovalent intermediate in which the inhibitor is virgin, C is the stable 1:1 complex of enzyme with inhibitor which is totally inactive, X and L* are two intermediates involving the modified inhibitor, and I* is the modified inhibitor in which the reactive-site peptide bond is hydrolysed [46, 47]. At low inhibitor and enzyme concentrations, the formation of C from E and I or I* is a simple second-order reaction and the following mechanism suffices for a description of the reaction:



where k_{on} and k_{on}^* are the second-order rate constants for the formation of C from E and I or I*, respectively. Similarly, k_{off} and k_{off}^* are first-order dissociation rate constants for the steps leading to the formation of E and I or I* from the stable complex C. The conversion of virgin inhibitor to the complex and back to the virgin form is faster than for the modified inhibitor. The equilibrium rate constant for hydrolysis is unity at or near neutral pH. The ideal inhibitors resemble ideal substrates in having very large $k_{\text{cat}}/K_{\text{m}}$ values; however, domain III of ovomucoids has the lowest possible k_{cat} and K_{m} values [48]. The structural mechanism of enzyme-inhibitor interactions has been extensively studied and reviewed by Bode and Huber [21]. Involvement of water molecules in the proteinase-inhibitor interactions has been shown [49].

The X-ray structures of serine proteinases with their inhibitors, including ovomucoid third domain, show that the reactive-site peptide bond is intact, indicating that the stable complex C is less than halfway on the path to hydrolysis of the reactive-site peptide bond [50–52]. The association of the modified inhibitor with the enzyme involves resynthesis of the reactive-site peptide bond.

The gene structure and evolutionary aspects of ovomucoid are discussed together with ovomucoid.

Ovo-inhibitor

The weak antichymotryptic activity exhibited by partially purified chicken ovomucoid prepared by the method of Lineweaver and Murray [2] was attributed to ovomucoid first identified and isolated by Matsushima [3]. It is present at around one-tenth the amount of ovomucoid; the actual level varies from species to spe-

cies. Like other egg white proteins, the synthesis and secretion of ovomucoid in oviduct is controlled by estrogen and progesterone, and is also stimulated by insulin and dexamethasone.

Ovomucoids from chicken, turkey, Japanese quail, penguin and ostrich have been isolated and studied in detail. The protein is immunologically related to the α_2 -proteinase inhibitor present in the serum and shares an identical sequence for the first 30 residues. It is possible that α_2 -proteinase inhibitor evolved first, as a plasma protein, and later adapted to the egg [53].

The carbohydrate content of ovomucoid is about 10%, much less than that of ovomucoid. Differences in sugar and sialic acid content are responsible for the heterogeneity observed in the ovomucoid preparations, which occur as five components on electrophoresis. Three glycosylation sites are present in chicken ovomucoid [54]. Like ovomucoid, ovomucoid is a specific inhibitor of serine proteinases such as trypsin, chymotrypsin, subtilisin, porcine elastase, proteinases K and F, and a fungal proteinase from *Aspergillus oryzae* [54, 55]. Proteinases from other mechanistic classes are not inhibited. Two independent binding sites are present for trypsin and chymotrypsin (or subtilisin) [56]. The two binding sites for porcine elastase overlap with one binding site each for trypsin and chymotrypsin but are separate from (do not overlap) that for the proteinase from *A. soyae*. The inhibition of porcine elastase is especially interesting, as ovomucoid inhibits the esterolytic, proteolytic and elastolytic activities of porcine elastase. In most cases of inhibition of porcine elastase, only the nonspecific proteolytic activity is inhibited.

Isolation

Crude ovomucoid prepared by the method of Lineweaver and Murray [2] has been used for the isolation of 0.6–0.7 g of ovomucoid from 1 l of egg white by Rhodes et al. [57] by subjecting crude ovomucoid to gel filtration and ion-exchange chromatography. This method is generally accepted.

Primary structure and physicochemical properties

Chicken ovomucoid is a 49-kD, 447-amino acid residue single polypeptide comprising seven homologous Kazal-type domains with covalently attached carbohydrate moieties [54, 57, 58]. About 51% of the amino acid residues are hydrophobic in nature. The amino acid sequence of the mature protein derived from cDNA [58] is identical to that determined from protein sequencing, except that an Asn at the twelfth position of the fifth domain was identified as Asp by protein sequencing. Since this amino acid is adjacent to Gly, it is possible that spontaneous deamidation of Asn to Asp

occurs in the protein sometime after its synthesis [58]. The putative signal peptide sequence as determined from cDNA studies conforms to the consensus identified by Watson [59].

It is not yet known whether all seven domains are independently active. However, at least five reactive sites are known to be present: two for trypsin, two for chymotrypsin, and one for porcine pancreatic elastase [56]. The P₁ amino acid residues (present in the reactive site) for the seven domains are Arg, Arg, Arg, Arg, Phe, Met and Met, respectively, indicating that the first four domains could potentially inhibit trypsin, the fifth chymotrypsin, and the sixth and seventh domains could inhibit both chymotrypsin and elastase [33]. The Japanese quail ovomucoid has six tandem homologous Kazal-type domains, the first three domains have potential reactive sites for the inhibition of trypsin, the remaining three for chymotrypsin and elastase [57].

The three-dimensional structure of ovomucoid is not yet known. However, the tertiary structures of bovine pancreatic secretory trypsin inhibitor (bPSTI) [60] and the third domains of Japanese quail, silver pheasant [30] and turkey [31] ovomucoids have been studied in detail. Similar elements of tertiary structure have been found in all the above cases, i.e. a triple-stranded antiparallel β -sheet and β -helix followed by a surface loop (see ref. 21). The reactive sites in these domains have similar structures and are not a part of the α -helices or β -sheets. It seems that individual domains of ovomucoid have a similar structure.

Chemical modification studies

Chemical modification of specific amino acid residues has been performed by several authors to elucidate involvement of a particular residue in the inhibition process. Nitrophenylsulfanylation of the single tryptophan residue and reductive methylation of the amino group of chicken ovomucoid do not result in any loss in the trypsin, chymotrypsin or subtilisin inhibitory activities [61]. However, modification of arginine with 1,2-cyclohexanedione was found to destroy the trypsin inhibitory activity. Reduced thioredoxin decreased the trypsin inhibitory activity of many proteins, including ovomucoid and ovomucoid [62].

Oxidation of methionine residues of ovomucoid with *N*-chlorosuccinimide resulted in selective loss of inhibitory properties [61]. While the trypsin inhibitory activity was not affected, 50% loss in chymotryptic activity and 100% loss in elastase activity were observed. The 50% loss of chymotryptic activity was attributed to the inactivation of one of its two inhibitory sites rather than a decrease in the binding constants of both sites [61].

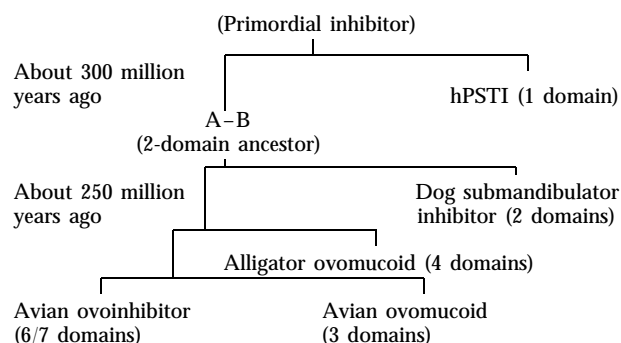


Figure 2. Line diagram tracing the evolution of multidomain Kazal inhibitors from a single domain primordial inhibitor.

Comparative structure of ovomucoid and ovomucoid genes

The chicken genome probably contains genes for at least four Kazal-type inhibitors, including ovomucoid and ovomucoid. Birds have only a single ovomucoid gene, and all ovomucoids studied are orthologous, which means they are products of the same gene in different species [58, 63, 64]. Homology between the three ovomucoid third domains can also be seen in intron placement. There is an intron at the start of each domain. The ovomucoid gene is present 9–23 kb upstream of the 5'-end of the ovomucoid gene. The gene is about 10.3 kb long and consists of 16 exons separated by 15 introns, the placement of introns for individual domains being similar to that in the ovomucoid gene. As in the case of ovomucoid, the first two exons of the ovomucoid gene code for the signal peptide and the first two amino acid residues of the mature protein, with the second (20-bp) exon coding for the C-terminal portion of the signal peptide and the first two amino acid residues of the mature protein. Each ovomucoid/ovomucoid domain is encoded by two exons. For each domain, one intron separates the codon of the first amino acid residue of the domain from the codon of the preceding domain. The first exons of ovomucoid second and fifth domains are three base pairs shorter than the first exons of the other domains in ovomucoid and ovomucoid. These deletions are in the region between the codons for the first and second cysteines.

The size and sequence of the corresponding introns in the ovomucoid and ovomucoid genes do not appear to be conserved, except for the intradomain introns of the third domain of ovomucoid and the seventh domain of ovomucoid, which are of similar sizes. The interdomain introns are about twice the size of intradomain introns.

Evolutionary aspects

The first and second domains of ovomucoid resemble each other more closely (and are called 'a' type) than the third domain (which is of 'b' type). Similarly, the

first six domains of chicken ovomucoid resemble each other more closely than the seventh C-terminal domain. Moreover, the first six domains of ovomucoid closely resemble the 'a' type domains of ovomucoid, and the seventh domain resembles the 'b' type domain of ovomucoid.

The six 'a' domains of ovomucoid are more closely related to the third domain than to any other domain, indicating that domain III has evolved at a slower rate relative to the other domains. From the sequence homology seen amongst the ovomucoid and ovomucoid 'a' type domains and amongst their 'b' type domains, it appears that both ovomucoid and ovomucoid have descended from a gene that coded for one 'a' and one 'b' type domain (fig. 2). The dog submandibular inhibitor has one 'a' and one 'b' domain, suggesting that the duplication event which formed the primordial two-domain inhibitor must have occurred prior to the mammalian-bird divergence, about 300 million years ago [63]. The addition of the present first domain of ovomucoid and the first five domains of ovomucoid occurred after the mammalian-bird divergence. A four-domain ovomucoid has been reported in alligator egg white [58], which has an N-terminal sequence homologous to the N-terminal sequence of chicken ovomucoid, implying that the duplication of the ovomucoid/ovomucoid two-domain ancestor must have occurred prior to the bird-alligator divergence (about 250 million years ago) [65]. The subsequent duplication of 'a' but not 'b' domains in ovomucoid and ovomucoid is probably because the 'a' domain boundaries correspond to exon-intron junction while the 'b' domain coding sequences are contiguous with the 3'-noncoding sequences.

From the sequence of a cDNA clone encoding human pancreatic secretory trypsin inhibitor (hPSTI), a single-domain Kazal serine proteinase inhibitor [66], there appears to be considerable homology with the ovomucoid and ovomucoid genes (about 45% nucleotide homology for signal peptide and 40–45% nucleotide homology to each of the 'a' and 'b' type domains of both ovomucoid and ovomucoid). It is possible that the ovomucoid-ovomucoid and hPSTI genes diverged prior to the duplication that generated the primordial 'a' and 'b' domains of multidomain Kazal inhibitors (fig. 2).

Ovomacroglobulin or ovostatin

Ovostatin is the third protein proteinase inhibitor present in avian egg white that has antitryptic activity. It was originally called 'line 18' or 'component 18' based on its movement in starch gel electrophoresis, and was later called 'ovomacroglobulin'. The protein has an amino acid composition similar to that of α_2 -macroglobulin from plasma. Trypsin inhibitory activity of ovomacroglobulin was reported by Kitamoto et al.

[4]. In 1983 Nagase and his group [67] identified a trypsin inhibitory protein in chicken egg white and named it ovostatin. In their subsequent paper [68] they acknowledged ovostatin to be the same as ovomacroglobulin, and suggested renaming component 18 or ovomacroglobulin as ovostatin, since this name describes both the source as well as function of the protein. A similar protein was isolated from the egg white of Cuban crocodile [69]. Ovostatin is synthesized in the oviduct and accumulates in the egg white. The protein has inhibitory activity towards trypsin, papain, subtilisin, rabbit synovial collagenase and thermolysin [4, 67, 68]. The native protein (MW 660,000) contains four subunits of equal molecular mass (175,000), each pair being disulfide linked. The dimers are held together by noncovalent interactions. A carbohydrate content of 0.28% has been estimated. Some of the physicochemical properties of this protein are given in table 1.

Isolation

Ovostatin can be prepared by gel filtration of egg white supernatant (obtained by homogenizing egg white in 1% sodium chloride and 1 M EDTA) on Sephadex G-200 column with further purification on Sepharose CL-4B and DEAE-cellulose columns [4]. The protein can also be purified by polyethylene glycol precipitation of egg white followed by chromatography on Ultragel AcA 34, DEAE-cellulose and Sephacryl S-300 columns [67].

Primary structure

Nagase and coworkers [67] have reported the sequence of the first 13 amino acid residues from the amino terminus as Lys-Glu-Pro-Glu-Pro-Glu-Tyr-Val-Leu-Met-Val-Pro-Ala. A comparison of this sequence with that of chicken α_2 -macroglobulin and human α_2 -macroglobulin shows considerable homology. It is quite possible that the structural genes for α_2 -macroglobulin and ovostatin may have evolved from a common ancestor. However, chicken α_2 -macroglobulin differs from ovostatin in the following properties:

1. Methylamine does not affect ovostatin, whereas it abolishes the inhibitory activity of α_2 -macroglobulin.
2. Electrophoretic mobility of the two proteins is different on 5% polyacrylamide gel electrophoresis.
3. There is no immunological cross-reactivity between these proteins.
4. Distinct mapping patterns of CNBr fragments of ovostatin and those of chicken α_2 -macroglobulin indicate that the two proteins have different amino acid sequences.
5. The NH₂-terminal sequences of ovostatin and α_2 -macroglobulin are distinct, although they show homology.

Inhibitory properties

Although the earlier preparations of ovostatin had shown complete absence of inhibitory activity, the same protein when isolated using different methods showed inhibitory activity against proteinases of all mechanistic classes [4, 67, 69, 70]. The absence of inhibitory activity in the former case was attributed to the method of protein isolation. Ovostatin inhibits the activity of trypsin by 20 and 50%, respectively, when BAPNA and casein are used as substrates. Maximum inhibition occurs at an ovostatin to trypsin molar ratio of 0.7 [4]. The caseinolytic activities of subtilisin BPN', papain and thermolysin are also inhibited; the maximum inhibition of 60% for papain and thermolysin occurs at a molar ratio of 0.8 to 1.0. Ovostatin binds to rabbit synovial collagenase and thermolysin at a molar ratio of 1:1 [67].

Inhibitory mechanism

The binding of ovostatin to rabbit synovial collagenase and thermolysin results in inhibition of proteolytic activities towards high molecular weight substrates only. Further, saturation of ovostatin with thermolysin prevents its subsequent binding to collagenase. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of bound ovostatin has shown that one of its subunits is cleaved into several smaller fragments upon binding to the enzyme, which prevents its subsequent binding to other enzymes. Ovostatin inhibits metalloproteinases to a greater extent than proteinases of other classes, in a manner similar to that elucidated for α_2 -macroglobulin. Hydrolysis of a peptide bond by the enzyme occurs in a susceptible region of the inhibitor, which triggers a conformational change in the ovostatin molecule. The enzyme becomes bound to ovostatin in such a way that larger molecules (enzymes/substrates/inhibitors) are sterically hindered from approaching the active site. Limited accessibility of soybean trypsin inhibitor (STI) to trypsin bound to ovostatin is possible because of its smaller size, and this leads to further inhibition of its inhibitory activity [67, 71].

Chicken cystatin

Barrett [72] proposed the name 'cystatin' for protein proteinase inhibitors of cysteine proteinases. Cystatin from chicken egg white was first isolated by Whitaker [5] and was found to inhibit ficin, papain and dipeptidyl peptidase, as well as cathepsins B, H and L [73]. The proteins of the cystatin superfamily are classified into three families (types 1, 2 and 3) on the basis of their molecular structure. Chicken cystatin is a type 2 cystatin. Like the other proteins of this family, it contains two disulfide bridges. It is present in egg white at a concentration of 80 $\mu\text{g/ml}$, as well as in serum and

muscle cells [73]. The protein is extremely heat stable and may be boiled for 30 min without loss of activity. However, freezing or freeze-drying results in loss of activity unless mixed with 20% glycerol or buffered at pH 7.5. Its stability towards alkaline solutions is questionable, as Fossum and Whitaker [5] reported loss of activity at pH 9.0 during boiling, while Anastasi et al. [73] reported high stability at alkaline pH.

Isolation

A two-step procedure has been described by Anastasi et al. [73] in which the inhibitor is obtained as two major, immunologically identical isoelectric forms. Cystatin is adsorbed batchwise on carboxymethylated papain-Sepharose and eluted at pH 11.5. Chromatofocussing on Mono-P column resolves the two forms in yields of 13 and 7% for forms I ($\text{pI} = 6.5$) and II ($\text{pI} = 5.6$), respectively. One of these is a full-length, 116-residue protein, while the other is a mixture of cystatins which are 8–9 residues shorter at the N-terminus. The chromatofocussing step can be replaced by ion-exchange chromatography [74]. Cystatin from duck egg white has been isolated by CM-papain affinity chromatography followed by size exclusion high-pressure liquid chromatography [75]. Five molecular forms were revealed upon electrophoresis with pI in the range 7.8–8.4. The method of Turk et al. [76] involves alkaline treatment, heat treatment at 65 °C, gel chromatography on Sephadex G-50 and ion-exchange chromatography on DEAE-Sephacel.

Structure of chicken cystatin

The complete amino acid sequence of chicken cystatin has been reported [77], and a molecular weight of 13,000 has been determined from the amino acid composition [74, 77]. Circular dichroism data and computer prediction of secondary structure show the presence of 20% α -helix with the remainder as β -structure. Preliminary X-ray crystallographic data on chicken cystatin showed crystals of trigonal space group P3₂1, with cell constants $a = b = 47.9 \text{ \AA}$, $c = 87.5 \text{ \AA}$, $\alpha = \beta = 90^\circ$, $\gamma = 120^\circ$, which contain one molecule per asymmetric unit [77, 78]. Bode et al. [79] solved the crystal structure of chicken cystatin by X-ray diffraction using multiple isomorphous replacement. The molecule consists of a five-stranded antiparallel β -pleated sheet wrapped around a straight five-turn α -helix. An appending segment of partial α -helical geometry is present. The first hairpin loop (comprising a highly conserved QVVAG or similar sequence) is present at one end of the sheet and is flanked by the N-terminal sequence and the second hairpin loop forming a wedge-shaped hydrophobic edge which is complementary to the active site of the enzyme.

Mechanism of inhibition

The great majority of cysteine proteinases of the papain superfamily, including ficin, papain, chymopapain, papaya proteinase III, actinidin, dipeptidyl peptidase I and cathepsins B, H, L and N are known to be inhibited by cystatin, while clostripain is inhibited only partially [5, 73, 80]. Papain, cathepsin B and dipeptidyl peptidase I bind to the same region of the cystatin molecule. Competing with the substrate, the interaction involves the active site of the enzyme and is strong, with a K_{assoc} of approximately 60 fM with papain [81, 82]. The binding of the inhibitor to the enzyme is reversible. The interaction between enzyme and cystatin is close enough to exclude molecules as small as iodoacetate from the reaction and is formed with minimal conformational adaptation of either protein [74, 82, 83].

Computer docking experiments indicate three regions of chicken cystatin that participate in the interaction with the enzyme – the segment of chain around Gly 9, the Gln-Leu-Val-Ser-Gly sequence at residues 53–57, and the loop around Trp-104 [80, 81, 83, 84]. The involvement of the two hairpin loops and the N-terminal region of cystatins in enzyme-inhibitor complex formation has been shown by several authors [85–90]. In the interaction of chicken cystatin with papain, the initially flexible N-terminal trunk loops over the catalytic Cys-25 residue of papain [21]. The QLVXG region of the first hairpin loop contributes most to the stability of the enzyme-inhibitor complex. The two disulfide bonds at Cys⁷¹-Cys⁸¹ and Cys⁹⁵-Cys¹¹⁵ of the chicken cystatin molecule help maintain the appropriate conformation of the inhibitor for its association with the enzyme [91]. The mechanism has been reviewed by Bode and Huber [21].

Müller-Esterl et al. [92] noticed that the sequence around Glu-Val-Val-Ala-Gly is highly conserved. The interaction of cystatin with papain involves cleavage of the Gly⁹-Ala¹⁰ bond; these are amongst the only three residues conserved in all known sequences of inhibitory cystatins [93]. The peptide bond of the conserved glycine is part of a substrate-like inhibitory site of the cystatin superfamily. Obviously, the cystatins have

evolved in the way most proteins do and are different from the serine proteinase inhibitors where hypervariability of the reactive site residues seems to be a normal phenomenon.

Ovalbumin

The function of ovalbumin in vivo is not known. It shares sequence homology with α_1 -proteinase inhibitor, antithrombin III and angiotensinogen, and can therefore be considered a member of the serpin family [94, 95]. Ovalbumin is specifically hydrolysed by elastase at a bond which is homologous with the reactive-site bond in α_1 -proteinase inhibitor [94]. Limited proteolysis of ovalbumin by subtilisin results in a nicked form of ovalbumin called plakalbumin, in which a hepta-peptide is released in several fragments as a consequence of proteolysis [94].

Proteinases can be classified into three broad classes by their effect on ovalbumin (fig. 3) – those causing extensive cleavage, those causing limited cleavage and those that do not cleave at all. Ovalbumin does not inhibit any of the serine proteinases tested thus far and can therefore be regarded as a nonfunctional serpin [95]. However, the specific cleavage of ovalbumin at the Ala³⁵²-Ser³⁵³ bond (the putative reactive site of the molecule) by subtilisin and porcine elastase is remarkable [96]. Estell and Laskowski [97] showed that enzymes exist which are homologous and closely related to enzymes that are strongly inhibited by protein inhibitors, and that instead of being inhibited they rapidly hydrolyse the reactive sites of many protein inhibitors. It is also possible that ovalbumin inhibits an as yet undiscovered or untested serine proteinase, or it had inhibitory activity which was lost during the course of evolution as it was no longer required.

Concluding remarks

The regulation of proteinases in tissues by endogenous inhibitors is essential. These proteases may be involved in extracellular matrix remodelling, cell differentiation, cell migration, protein folding etc. Although the involvement of protein proteinase inhibitors of egg whites in warding off microbial infection and embryonic tissue differentiation has been suggested, it remains to be proven. In spite of the extensive work done on ovomucoids, the enzymes physiologically inhibited by it are not known. This is also true for the other egg white inhibitors.

Acknowledgements. Financial assistance from the University Grants Commission, Delhi, in the form of an NET Scholarship to Indu Saxena is gratefully acknowledged.

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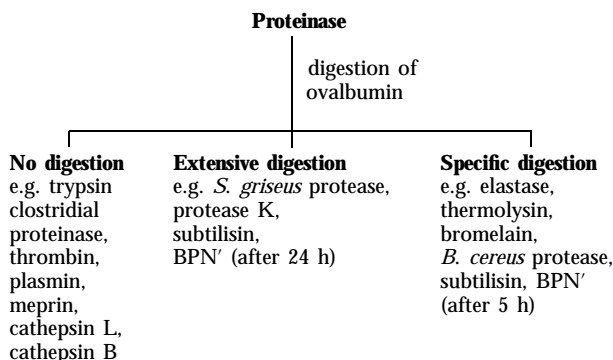


Figure 3. Classification of proteinases on the basis of their action on ovalbumin.

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