Action of Proteolytic Enzymes on Chromosomes in vivo

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Since the discovery of residual proteins in the chromosomes, almost three decades have past, yet their role in chromosome organization and function is only partially understood. One methodology to elucidate the role of proteins in chromosome structure would be to study directly the effects of specific proteolytic enzymes on various constituents of the chromosomes in living cells. Such studies have been carried out with DNase on the microsporocytes of *Tradescantia* by Bal and Kaufmann (1959) and on salivary gland of *Drosophila* and testicular cells of grasshopper by Dutt and Kaufmann (1959). So far as the proteases are concerned early works of Kaufmann and his group dealt with their effect on fixed cells. Maio and Schildkraut (1966) have carried out studies on the effect of pepsin and trypsin on the isolated chromosomes and Al-Aish (1971) on the effect of pronase on cultured lymphocytes.

The present investigation deals with the direct action of trypsin, pepsin and pronase on the living chromosomes of *Rhoeo* microsporocytes. It has been repeatedly shown from this laboratory (De and Ghosh 1967, 1968a, 1968b) that plant chromosomes contain a protein which is susceptible to collagenase. Hence, the action of the enzyme collagenase has also been investigated. The study reveals that indeed the nonhistone residual protein may be implicated in the maintenance of the chromosome morphology as a ligand macromolecule and the histones may be responsible for chromosome coiling.

Methods

A special technique had to be developed to study the effect of enzymes on living chromosomes. The standard microscope slides were modified so that the slides are provided with two oblong grooves placed diagonally. Strips of 'para-film' are attached at the two sides of the slide so that the cover glass rests on a slightly elevated position, free flow of fluid is ensured and the microsporocytes are not crushed. Under this condition, the chromosomes of the microsporocytes can be observed very well with the aid of a phase contrast microscope continuously throughout a treatment and fluids can be changed under constant observation. In all the experiments microsporocytes at metaphase I from the selected anther lobes of *Rhoeo discolor* were gently squeezed out on a slide, immediately a drop of the

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desired solution was placed and covered with a cover glass. When desired the cells were stained with acetocarmine or by Feulgen method. A series of photomicrographs were taken to record the various effects.

The four proteolytic enzymes used are trypsin (Type III, 2×cryst., Sigma), pepsin (2×cryst., Sigma), pronase (nuclease free, Calbiochem) and collagenase (Type I, Sigma). The enzymes were dissolved in 0.05 M Na_2HPO_4 - KH_2PO_4 buffer at pH 6.0, 0.02 M NaCl, 0.02 N HCl at pH 1.6 and 0.2 M sucrose at pH 6.5, in addition to distilled water at pH 6.5. For all the various enzyme treatments respective controls were maintained under identical conditions in the solution without the enzyme. The treatments were carried out at various temperatures e.g. 22°C, 26°C, 37°C. Preliminary studies indicated that the lower temperature only slowed down the action of the enzymes and did not produce any qualitative change in the effects of the enzymes as compared to higher temperatures. Hence all enzymes treatments were carried out at 37°C.

Experiments and results

Preliminary investigations were carried out with the enzymes dissolved in a solution in which they are known to show optimum activity. Thus trypsin was dissolved in phosphate buffer at pH 6.0, pepsin in 0.02 N HCl at pH 1.6 pronase in 0.2 M NaCl and collagenase in distilled water. All the enzymes showed various effects on metaphase chromosomes (Fig. 1) and surprisingly the control solutions showed some effects. Even distilled water at pH 6.5 at 37°C and also at 22°C caused a little despiralisation and swelling of chromosomes within 25 minutes. Hence a solvent had to be found out which by itself would have no effect on the chromosome and would yet retain the activity of the enzymes when dissolved in it. It was noted that all the various solutions tried as the medium for the enzymes changed the osmotic balance of the cell. Hence, to maintain the osmotic pressure of the cell, sucrose solution was tried out as the solvent for the enzymes and it was observed that 0.2 M solution of sucrose in water was isotonic with cell sap and had no or very little effect on the chromosomes and yet maintained the activity of the enzymes. The results are presented in Table 1.

Table 1. Period of treatment with various solvents producing successive changes in chromosome morphology

Solvents	Uncoiling	Swelling	Dispersion
Water	15 min	25-35 min	> 45 min
NaCl soln.	15 min	25 min	> 60 min
HCl soln.	11-15 min	40 min	> 60 min
Buffer	15 min	25-35 min	> 45 min
Sucrose soln.	90 min	—	—

It is obvious that all the solvents are capable of producing gentle morphological changes of the chromosomes in the sequence of uncoiling, swelling, and final spreading over the whole nucleoplasm giving an interphase-like appearance. The sucrose solution produced least effect, causing only little uncoiling and swelling after 90-

120 min. Even prolonged treatment for 4 hours did not produce any swelling or spreading. Hence for the present study, each enzyme was dissolved in the sucrose solution, in addition to its standard solvent.

Figs. 1-9: Photomicrographs of acetocarmine stained *Rhoeo* microsporocytes showing the effects of the proteolytic enzymes (in sucrose solution) on the chromosomes. 1, control cell showing early anaphase I chromosomes. 2, 3 and 4, treated with trypsin (0.125 mg/ml.): showing thinning of chromosomes after 1 min, uncoiling and swelling after 4 min and dissolution after 7 min respectively. 5, 6 and 7, treated with pepsin (4 mg/ml.): showing gentle swelling of chromosomes after 7 min, clumping after 11 min and spherule formation after 45 min respectively. 8 and 9, treated with collagenase (4 mg/ml): showing despiralization of chromosomes after 11 min and vacuolation after 45 min. Single arrows indicate vacuoles and double arrows indicate fibrous component of chromosomes.

It was soon realised that the identical concentration of the enzymes failed to produce comparable results in a given period of time. Hence to study the effects of the enzymes within a practical limit of 4 hours of continuous microscopic observation, the concentrations were varied. Thus trypsin was used as 0.125 mg/ml, pepsin as 4.0 mg/ml, pronase as 1 mg/ml and collagenase as 4.0 mg/ml. It was also noted that the sequence of changes in the morphology of chromosomes produced by a given enzyme was more or less same irrespective of its concentration, solvent medium or temperature. The details of the effect of the enzymes on the chromosome morphology of the living cells are given below.

Effect of trypsin

Within few minutes of the application of the enzyme the first effect could be recorded by a detectable reduction of the diameter of the chromosomes (Fig. 2) which was followed by gradual despiralisation. Longer treatments produced swelling of the chromosomes with concomitant loss of stainability (Fig. 3). Finally the metaphase chromosomes were found to be dissolved and the fine fibres were barely detectable (Fig. 4). The results are summarised in the Table 2.

Table 2. Period of treatment with trypsin producing successive changes in chromosome morphology

Enzyme solvent	Thinning	Uncoiling	Swelling	Dissolution
Buffer	—	4 min	5–7 min	> 11 min
Sucrose soln.	1 min	2–3 min	4–6 min	> 7 min

Effect of pepsin

Within 10 minutes of treatment with pepsin the chromosomes swelled and clumped together (Figs. 5 and 6) and by about 15 minutes chromosomes took the appearance of spherules. These spherules were more or less spherical vacuole-like bodies and were not stained by carmine. With increasing time these spherules increased in size and became highly refractive (Fig. 7). They did not undergo further change even after four hours of treatment. The results are summarised in Table 3.

Table 3. Period of treatment with pepsin producing successive changes in chromosome morphology

Enzyme solvent	Swelling	Clumping	Spherule initiation	Complete spherulation
HCl soln.	7 min	10 min	15 min	> 25 min
Sucrose soln.	7 min	11 min	15–35 min	> 45 min

Effect of pronase

Irrespective of the solvent of the enzyme, the first effect of pronase as thinning of chromosomes was evident within first minute. This was followed by uncoiling and swelling. By 4 to 7 minutes the metaphase chromosomes were completely dissolved in the nucleoplasm producing a mass of barely visible fine fibres which

were poorly stainable. The appearance did not change even after prolonged treatment. The results are summarized in Table 4.

Table 4. Period of treatment with pronase producing successive changes in chromosome morphology

Enzyme solvent	Thinning	Uncoiling	Swelling	Dissolution
Water	← 1-2 min →		3 min	> 4 min
NaCl soln.	1 min	← 1-2 min →		> 3 min
Sucrose soln.	1 min	2-3 min	4-5 min	> 7 min

Effect of collagenase

The sequence of events that took place after treatments with collagenase is very distinctive. After about 5 minutes the chromosomes were uncoiled (Fig. 8) followed by swelling and clumping. Moreover fragmentation of the chromosomes was also noticed. After about 20 minutes the disorganised chromosomal material appeared to segregate in two separate components: i) a deeply staining fibrous material surrounded by ii) a number of small vacuoles. No doubt the vacuoles were formed from the dissolved chromosomal materials. With progression of time the deeply staining fibrous material underwent further degeneration with simultaneous increase in volume of the vacuoles leading to fusion. The final picture reached after about three hours was that of a highly swollen cell with a central large poorly stainable vacuole surrounded by a thin layer of fibrous material and the cytoplasm became appressed to the cell wall (Fig. 9). It may be stated here that the effect of the enzyme collagenase showed a noticeable difference depending on the solvent. The sucrose medium showed the ultimate dissolution within about 10 minutes whereas other solvents took 45 minutes or more. The results are summarised in the Table 5.

Table 5. Period of treatment with collagenase producing successive changes in chromosome morphology

Enzyme solvent	Uncoiling	Clumping	Initiation of vacuolation	Complete vacuolation
Water	11 min	15 min	20-30 min	> 45 min
NaCl soln.	—	15 min	25-35 min	> 45 min
Buffer	—	15 min	25-35 min	> 45 min
Sucrose soln.	1-2 min	3 min	4 min	> 7 min

Discussion

It came as a surprise to us that the various control solvents in which the enzymes are usually dissolved for optimum activity, are capable of inducing despiralization of the chromosomes. This may be due to a change of its osmotic pressure, since isotonic solution can greatly minimize the effect. The maximum effect produced by these solvents is the dispersion of the chromosomes to give an interphase-like appearance of chromosome in which distinct fibres are noted. On the contrary, the maximum effect produced by the enzymes goes far beyond that, so that the

chromosomes are dissolved and barely the fine fibres are detectable. These effects thus conclusively show that the high molecular weight enzymes can indeed enter a cell, thus negating the contention by Kaufmann and Das (1954) that pepsin and trypsin are incapable of penetrating the cells.

All the proteolytic enzymes used here are capable of causing dissolution of the chromosome, but through different intermediate steps, final step being different only in case of collagenase where the segregation of chromosomal components in the form of fibrous matter and vacuole, is noted. Moreover, the efficacy of the enzymes for comparable effects vary widely as denoted by 0.125 mg/ml for trypsin and 4 mg/ml for pepsin or collagenase. All these are expected on the basis of the specificity of the enzyme and the state of its substrate.

Trypsin has a narrow specificity of endopeptidase activity, freeing the carboxyl groups of lysyl and arginyl residues in the peptide chain, though such cleavage is inhibited where the next residue is prolyl, aspartyl or glutamyl. Thus it has been shown that in isolated nuclear suspension, about 70% of total histone is degraded and released, while the losses of nonhistone protein was comparatively small (Allfrey and Mirsky 1964). Maio and Schildkraut (1966) reported that trypsin disorganizes the isolated chromosome. The primary thinning of chromosomes by trypsin in the present report indicates the removal of proteins which represent the matrix and are probably basic in nature. Subsequent dissolution after prolonged treatment indicates further breakdown of chromosomal proteins. Even on fixed chromosomes, lengthening of the chromosomes with concomitant unraveling has been reported by Wolff (1969). The conclusion is drawn that the dissolution of the chromosome by trypsin is caused by removal of histones. This is supported by our previous studies that indeed the chromosomes loose a considerable amount of histones when they are despiralized by HNO_3 -fumes (De, Mitra and Nasar 1973).

Pepsin is also an endopeptidase acting on peptide link between dicarboxylic aminoacid and an aromatic aminoacid. Since, aromatic aminoacids are few in histones, it acts chiefly on nonhistones. Although Maio and Schildkraut (1966) failed to detect any action of pepsin on isolated chromosome, a distinct phenomenon called as spherule formation has been noted in living chromosomes of *Rhoeo*. These spherules are identical to spherule formation in *Tradescantia* meiotic metaphase chromosomes when treated with DNase (Bal and Kaufmann 1959). The same phenomenon has also been observed on salivary gland chromosomes of *Drosophila* and testicular cells of grasshopper (Dutt and Kaufmann 1959) by DNase. The similarity of effects of pepsin and DNase are intriguing and on the contention that DNA represents the chromosomal axis, it is tempting to conclude that non-histone proteins may have a similar role in maintaining chromosome structure, and forms an 'inner' component.

Pronase acts both as an endopeptidase and as an exopeptidase and has wide substrate specificity causing almost complete breakdown of proteins to aminoacids. Maio and Schildkraut (1966) reported that on isolated chromosomes, the enzyme causes expansion and lengthening of chromosomes finally to almost dissolution. Al-Aish (1971) studied the effect of the pronase on chromosomes of human lymphocytes in culture. He found that the enzyme produced stickiness, loss of staining

ability, incipient banding and finally disintegration. The present results are in consonance with these observations. Hence it can be concluded that pronase acts on all proteins including histones and nonhistones.

According to Nagai (1959) the enzyme collagenase splits specifically the peptide bond between X-glycine in polypeptides which contain the sequence proline-X-glycine-X (in which hydroxyproline may replace proline and X may be any amino-acid). Hence, collagenase may attack some other proteins other than collagen. The effect of collagenase on chromosomes is unique in that in certain cases it can cause fragmentation in addition to despiralization unlike other enzymes. Thus it is tempting to conclude that collagen-like protein may work as a ligand in the chromosomes.

In conclusion, it may be stated that trypsin acts chiefly on histones which forms the 'outer' part of the chromosomes and which may be responsible for chromosome coiling. Pepsin acts on the nonhistone protein which may be located in the 'inner' part of the chromosome. Some fraction of the inner protein may be collagen-like in its susceptibility to collagenase.

Summary

The action of four proteolytic enzymes viz. trypsin, pepsin, pronase and collagenase, on the chromosomes of living microsporocytes of *Rhoeo discolor* was studied. Continuous observation for a period of 4 hours indicated that the solvents in which the various enzymes are dissolved had a little effect on the chromosome morphology, which can be minimized by making the solution isotonic with sucrose. All the enzymes are finally capable of producing distinct dissolution of the chromosomes, but the intermediate steps towards dissolution are different. On the basis of specificity of the enzymes, it has been concluded that histones are responsible for coiling of chromosomes and nonhistones play an important part as a ligand macromolecule.

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