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POLYPHENOL INTERACTIONS: ASTRINGENCY AND THE LOSS OF ASTRINGENCY IN RIPENING FRUIT*

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Key Word Index—Polyphenols; tannins; β-glucosidase inhibition; protein-polyphenol complexation; astringency.

Abstract—The inhibition of the enzyme β-glucosidase by natural polyphenolic substrates is described. The kinetic data suggest that the pattern of inhibition which is initially observed most closely resembles that of the classical non-competitive type. The system has also been employed to test the ability of other substrates, such as proteins, various polysaccharides, α- and β-cyclodextrins and caffeine, to disrupt the association of polyphenols with β-glucosidase. The results are utilized to put forward a hypothesis for the loss of astringency which typically occurs in some fruit upon ripening.

INTRODUCTION

Polyphenols (*syn* vegetable tannins [2])–(proanthocyanidins and esters of gallic acid and hexahydroxydiphenic acid) constitute a distinctive group of higher plant secondary metabolites. Their uniqueness lies not only in their polyphenolic character and the range of molecular weights they possess but also in their ability to complex strongly with proteins, certain types of polysaccharide and carbohydrate, nucleic acids and alkaloids—indeed the 'umbrella' nomenclature vegetable tannin for this group of substances derives historically from these fundamental properties. Studies of these characteristics are not only of intrinsic scientific interest, but they are also of considerable practical significance. Thus the taste, palatability [3], nutritional value [4, 5], the pharmacological and physiological effects of plant materials [6] and their microbial decomposition [7,8] are all properties which are substantially influenced or modified by the polyphenols which they contain.

As a sensation of taste, that of astringency in fruits and beverages is a subtle and distinctive one. In so far as understanding the underlying mechanisms of the astringent response is concerned then attention inevitably focuses upon the polyphenols in the plant tissue since, according to Bate-Smith [9, 10] the primary reaction is that of cross-linking of proteins and mucopolysaccharides in the mucous secretions by polyphenolic molecules (Fig. 1). Loss of astringency is one of the principal changes which occur during the ripening of many edible fruits. Although some astringent fruits are reputed to show a diminution of soluble polyphenols upon ripening [11, 12], many others do not [11, Haslam, E., unpublished observations]. In such cases it is not immediately obvious how ripening and loss of astringency are interrelated. The work presented here discusses the role which substrates possessing the ability to disrupt polyphenol–protein complexation may have upon the astringent reaction and the significance such molecules may have on the loss of astringency in ripening fruit.

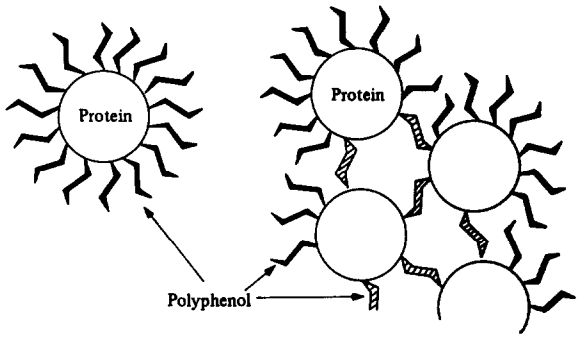

Fig. 1. Protein precipitation by polyphenols—the astringent response.

RESULTS AND DISCUSSION

Various quantitative methods have been described to determine the propensity of polyphenols to associate reversibly with proteins and these include equilibrium dialysis [13], microcalorimetry [13] and various precipitation techniques [14–16]. It is well known that polyphenols inhibit enzymes [17] and this property has now been exploited in order to measure quantitatively not only the variations in the affinity of individual proteins for different polyphenols but also the extent to which other substrates may disrupt and hence modify this binding capacity. Based on earlier observations [15, 17] and its availability, the enzyme β-glucosidase (E.C. 3.2.1.21) was selected for detailed study.

β-Glucosidase activity was assayed continuously at λ 360 nm using *p*-nitrophenyl-β-D-glucopyranoside (Fig. 2) as substrate (pH 5.0, acetate buffer, $K_M = 5.5 \times 10^{-3}$ M). Kinetic measurements were made in the

*Polyphenol Interactions. Part 3: for Part 2; see reference [1]

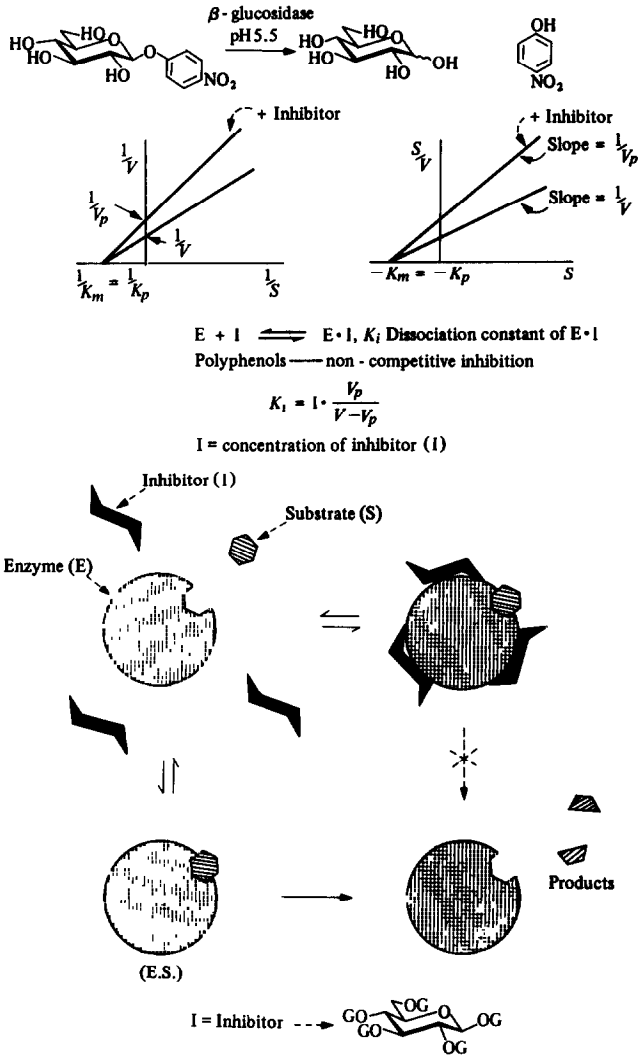

Fig. 2. Non-competitive enzyme inhibition with polyphenolic substrates.

presence of various polyphenolic substrates, β-1,3,6-tri-*O*-galloyl-D-glucopyranoside (**1**), β-1,2,3,6-tetra-*O*-galloyl-D-glucopyranoside (**2**), β-1,2,3,4,6-penta-*O*-galloyl-D-glucopyranoside (**3**), β-1-*O*-galloyl-2,3:4,6-*O*-bis-*S*-hexahydroxydiphenoyl-D-glucopyranoside (**4**)-casuarictin, rugosin-D [18, 19] (**5**) and sanguin H-6 [18, 20] (**6**) used in earlier related work [13]. In all cases the kinetics observed were most closely correlated with the classical pattern of non-competitive inhibition in which polyphenol and substrate are assumed [21] to bind simultaneously to the enzyme. In the simplifying case assuming Michaelis–Menten kinetics K_M remains unaffected, the [enzyme-inhibitor-substrate] complex does not react, and K_i may be determined and used as a quantitative measure of the affinity of the polyphenolic inhibitor for the enzyme β-glucosidase (Fig. 2, Table 1) low values of K_i indicate a relatively strong affinity for the protein, high values a correspondingly weak affinity for protein. The values of K_i obtained for different polyphenolic substrates and the observed trends (Table 1) broadly exemplify the same characteristics of molecular structure in the polyphenolic substrate which lead to an enhanced capacity to bind to protein which were identified in earlier work [13]–namely, increasing molecular size, conformational flexibility and low water solubility. The presumed mechanism of non-competitive enzyme inhibition is worthy of brief comment. The dominant feature is assumed to be that in which the polyphenolic substrate complexes to the protein surface cross-linking and thereby 'locking' adjacent regions of the enzyme structure such as to prevent the necessary conformational changes which must ensue for catalysis to occur. Whether there is a clearly defined specificity for association of polyphenolic metabolites at particular sites in a protein molecule is not yet entirely clear. However if one assumes, on the basis of present evidence, this to be a process possessing a degree of randomness then complexation at or near the enzyme active-site might also be reasonably anticipated. This would then give rise to direct competitive inhibition of the enzyme. From the present kinetic data it must be pre-

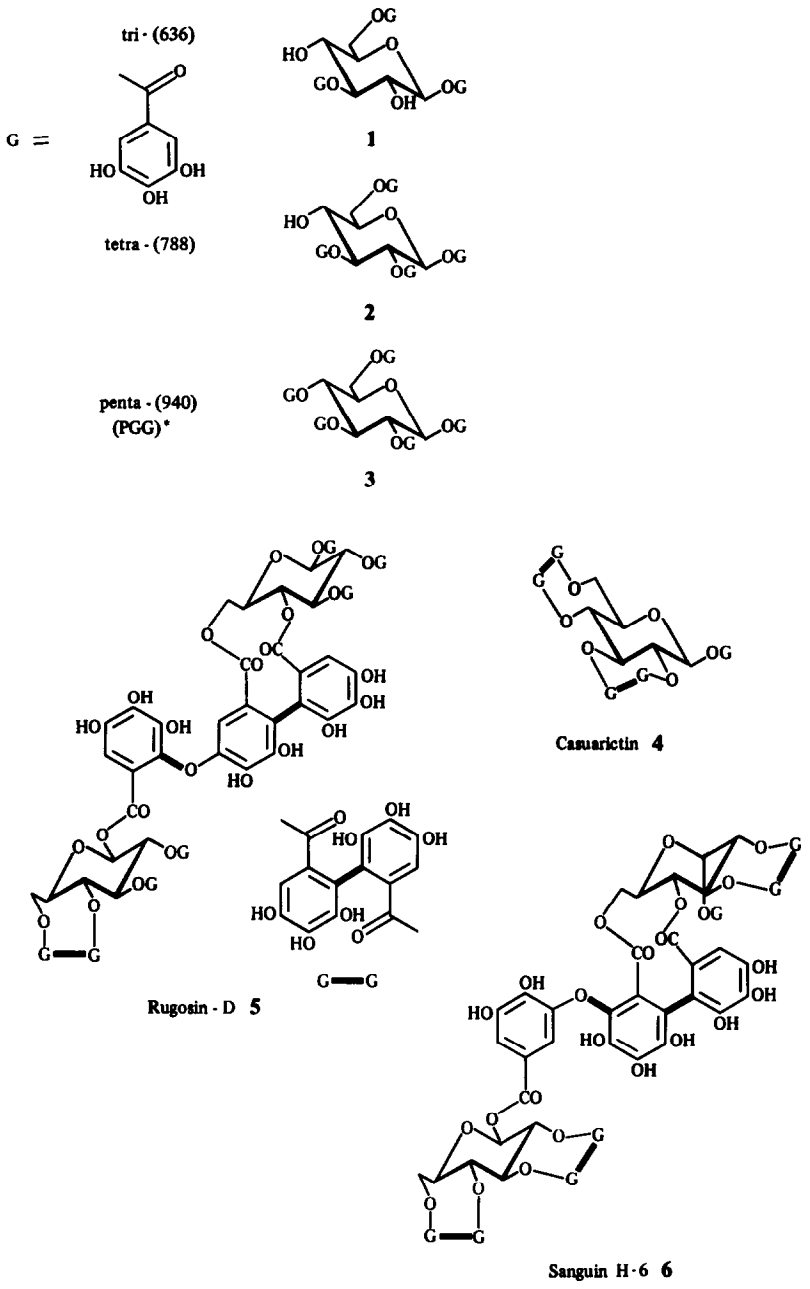

Table 1. Reversible complexation of polyphenols with proteins

Polyphenol	Molecular weight	β-Glucosidase inhibition $K_i(10^{-4}M)$	BSA $-\Delta G^{\theta,tr}$ (kj/mol)*	Haemoglobin precipitation (relative astringency)†
β-1,2,6-Tri-*O*-galloyl-D-Glucose (1)	636	10.8	0.9	0.20
β-1,2,3,6-Tetra-*O*-galloyl-D-glucose (2)	788	2.50	9.1	0.58
β-1,2,3,4,6-Penta-*O*-galloyl-D-glucose (3)	940	0.85	26.9	1.0
Casuarictin (4)	936	1.57	–	–
Rugosin-D (5)	1874	0.08	58.7	2.4
Sanguin H-6 (6)	1870	0.40	11.3	

* $-\Delta G^{\theta,tr}$ Free energy of transfer of the protein BSA from an aqueous solution to an aqueous solution containing the polyphenol [13,31].

†Relative astringency by haem analysis [32]. Values related to (3) as standard 1.0 [33].

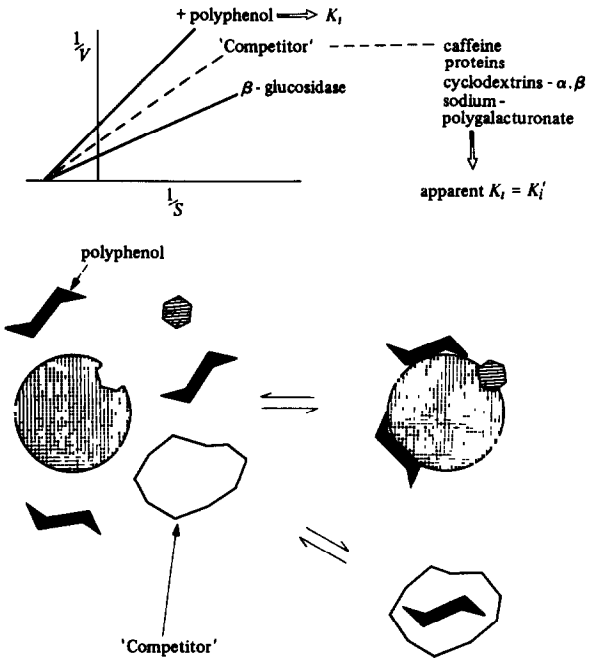

Fig. 3. Relief of non-competitive enzyme inhibition—the disruption of protein—polyphenol complexation.

Table 2. Relief of enzyme inhibition caused by the disruption of protein–polyphenol complexation

Competitor	K_i' (observed for β-1,2,3,4,6-Penta-*O*-galloyl-D-glucose*)
Caffeine	
(i) 0.995×10^{-3} M	0.95×10^{-4} M
(ii) 1.49×10^{-3} M	1.17×10^{-4} M
(iii) 1.99×10^{-3} M	1.28×10^{-4} M
Bovine serum albumin	
(i) 0.826×10^{-3} mg/ml	1.17×10^{-4} M
(ii) 2.07×10^{-3} mg/ml	1.84×10^{-4} M
Sodium polygalacturonate	
(i) 0.444 mg/ml	1.21×10^{-4} M
(ii) 0.889 mg/ml	2.22×10^{-4} M
α-Cyclodextrin	
(i) 0.499×10^{-3} M	1.12×10^{-4} M
(ii) 0.998×10^{-3} M	1.26×10^{-4} M

*β-Glucosidase- 4.15×10^{-3} mg/ml and β-1,2,3,4,6-penta-*O*-galloyl-D-glucose- 0.50×10^{-4} M. Polyproline (M_r 30000 and 7000) at concentrations of up to 4.00×10^{-3} mg/ml had no effect on the K_i (observed) for β-1,2,3,4,6-penta-*O*-galloyl-D-glucose.

Caffeine (5.60×10^{-3} M and 8.00×10^{-3} M) gave values of K_i (observed) for β-1,3,6-tri-*O*-galloyl-D-glucose (4.032×10^{-4} M) of 16.9×10^{-4} and 17.8×10^{-4} M respectively.

sumed that, in the case of β-glucosidase, this initially makes a relatively small contribution to the overall pattern of inhibition.

This system has been employed additionally to determine—in a quantitative manner—the ability of other substrates to disrupt the capacity of polyphenols to bind to the protein β-glucosidase. Previous work has shown, for example, that polysaccharides (particularly those such as amylose which can develop secondary structure containing hydrophobic cavities [13]), polyamides (eg. polyvinylpyrrolidone [22]) non-ionic detergents [17, 23], polyethylene oxides [17, 24], α and β-cyclodextrins [25] and alkaloids, such as caffeine [26] and cinchonine [27], associate strongly with polyphenolic substrates. Several of these substances have been tested in the assay and their capacity to compete for the polyphenolic substrates measured by their relief of inhibition of β-glucosidase activity using the polyphenol β-1,2,3,4,6-pentagalloyl-D-glucopyranoside (3) (Fig. 3) as a standard. The relief of inhibition is represented as a change in the typical inhibitor constant K_i for (3) to K_i' (Table 2).

Variety, maturity and climate are known to influence the astringency of fruit and present evidence suggests that each fruit must be considered as a separate case rather

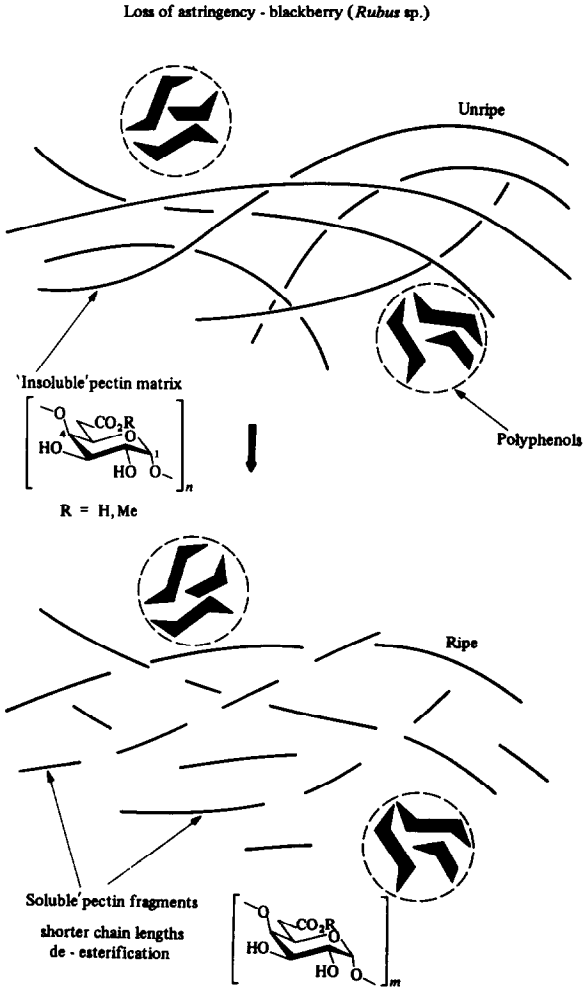

Fig. 4. A schematic molecular interpretation of the loss of astringency in blackberry fruit (*Rubus* sp.).

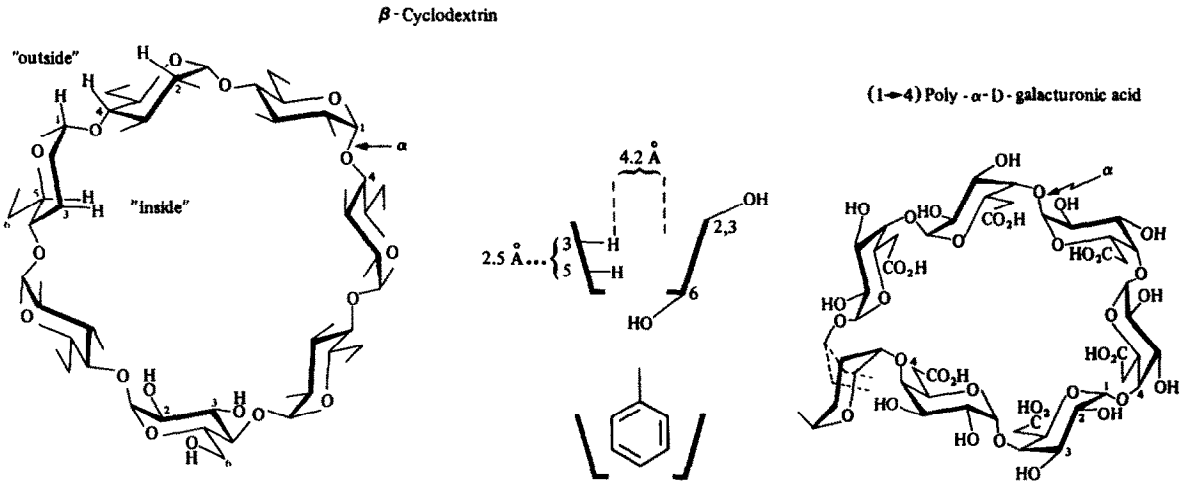

Fig. 5. (a) β-Cyclodextrin and (b) (1→4) poly-α-D-galacturonic acid—molecular cavities.

than there being an all-embracing explanation for the loss of astringency on ripening. Matsuo and Itoo [12] have suggested for example that in persimmon (*Diospyros kaki*) loss of astringency is due to the immobilization of the tannin caused by reaction with acetaldehyde produced during the ripening process. The principal polyphenols in fruit of *Rubus* sp. are based on gallic and hexahydroxydiphenic acid [18] and comprise compounds such as peduncalagin and the dimer sanguin H-6 (6) [18, 20]. During the ripening process and in the ripened fruit themselves there is no *substantial* change in the composition or the concentration of these polyphenolic metabolites. It is well known that in the ripening process the celllular structure of many fruit is modified, softening occurs and this is accompanied by release of smaller soluble pectin fragments in which de-esterification has also occurred [28], (Fig. 4), on some of the methyl-D-galacturonate residues. The ability of sodium poly-D-galacturonate to disrupt the binding of polyphenols to the protein β-glucosidase has been demonstrated in this work (*vide supra*) and this most probably results from the ability of the polysaccharide to develop a secondary structure in solution containing hydrophobic pockets somewhat similar to the cyclodextrins [29], (Fig. 5). It thus seems highly probable that in some fruit one cause of the loss of astringency which occurs upon ripening may well be the production of significant amounts of water soluble fragments of the pectin structure as the cellular structure softens [30]. These soluble polysaccharides are presumably of appropriate structure and produced in sufficient quantities to effectively compete with the mucal polysaccharides and proteins for the polyphenolic substrates when the fruit is tasted in the mouth and thus lead to a modification of the astringent response. Further work is in progress on this problem and also upon the development of the β-glucosidase system to investigate the selectivity of protein–polyphenol interactions and the complexation of polyphenols with other substrates.

EXPERIMENTAL

Materials. β-Glucosidase from sweet almonds was purchased from Koch-Light (Activity ~ 1000 units/mg). *p*-Nitrophenyl-β-D-glucoside (PNG) was synthesized. Polygalacturonic acid sodium salt, bovine serum albumin (BSA) α and β-cyclodextrin were obtained from Sigma. Polyphenols were obtained by previously described methods [13]; 1,3,6-tri-*O*-galloyl-β-D-glucopyranose (1) by partial hydrolysis of chebulinic acid; 1,2,3,6-tetra-*O*-galloyl-β-D-glucopyranose (2) from *Bergenia* sp.; 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose (3) by methanolysis of tannic acid; 1-*O*-galloyl-2,3:4,6-*O*-bis-*S*-hexahydroxydiphenoyl-β-D-glucopyranoside (4) and sanguin H-6 (6) from Raspberry leaves, rugosin-D from *Rosa* sp. and *Filipendula ulmaria*.

β-Glucosidase assay (with P. N. Goulding). Reaction mixtures used for the determination of K_m and K_i were prepared as follows. Solutions of PNG and of β-glucosidase were prepared in 0.2 M acetate buffer pH 5.0. Each reaction mixture was of total volume 9 ml, consisting of 2.0 ml polyphenol solution, 0.5 ml enzyme solution to give a concentration of 4.10×10^{-3} mg/ml and a volume of PNG solution to give a concentration of 1.5–9.5 $\times 10^{-4}$ M. After a 10 min preincubation at 30°, the reaction was initiated by addition of enzyme solutions (0.5 ml), and assayed at one min intervals by measuring the absorbance at 360 nm at 300°. Initial velocities were calculated from the gradient of the linear portion of the reaction curve (product formation vs time) for each PNG concentration, and these were plotted by a method most suitable for calcn of K_i or K_m. Typical values for 50% inhibition under these conditions were β-1,3,6-tri-*O*-galloyl-D-glucose (1) 8.0×10^{-4} M; β-1,2,3,6-tetra-*O*-galloyl-D-glucose (2) 2.11 $\times 10^{-4}$ M; β-1,2,4,6-penta-*O*-galloyl-D-glucose (3) 0.67 $\times 10^{-4}$ M; Casuarictin (4) 1.49×10^{-4} M; rugosin-D 0.65 $\times 10^{-5}$ M; sanguin H-6 0.29×10^{-4} M.

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