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Short Communications

Lactoperoxidases of Monkey Saliva

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Studies of lactoperoxidase activity from various sources, including bovine milk,[1–4] Harderian and lacrimal glands of the cow [5] and the saliva of man, cow and pig,[6] have previously been reported. The present communication provides preliminary information about the purification and properties of the lactoperoxidase of monkey parotid and submandibular saliva with preliminary results of experiments on lacrimal fluid.

The animals used in the investigation (*Macaca mulatta* and *M. fascicularis*) have been described in a previous report.[7] The saliva and lacrimal fluid samples studied in this communication were collected as part of an experiment to determine the effects of xylitol on salivary composition.[7] The study was carried out on five *M. mulatta* and ten *M. fascicularis* monkeys. After the accomplishment of the diet study [7] all parotid and submandibular saliva samples of each species were pooled for the present investigation. Lacrimal fluid samples obtained from *M. fascicularis* were also pooled. Details of the animals and their diets were previously published.[7]

Saliva samples (0.1 ml to 1.2 ml aliquots) were obtained by direct cannulation of salivary ducts following pilocarpine stimulation, and lacrimal fluid was collected by means of a micropipette from the conjunctival sacs while the animals were under anaesthesia.[7] The saliva was stored in sealed plastic containers for 6 to 8 months at −20 °C prior to the present studies. Storage did not reduce the enzyme activity to any detectable extent. Lactoperoxidase activity was determined according to the guaiacol method.[8] Details of these and other methods, chromatographic procedures and reagents have been published previously.[9,10] The units (U) of enzyme activity were calculated as the ratio of 60 s to the reaction time in seconds to reach an extinction of 0.050 in a reaction mixture described previously.[8–10] It was shown that there was a linear relation between the reaction time in seconds and enzyme activity if the specific activity was higher than one enzyme unit per mg protein. This criterion was followed in all kinetic experiments. In some chromatographic experiments this practice was not conceivable.

CM-Cellulose chromatography of parotid saliva of *M. mulatta* yielded repeatedly a single sharp peroxidase peak which coincided with the main protein peak. When the active fractions resulting from CM-cellulose chromatography of parotid saliva were pooled and the resulting enzyme solution was passed through a Sephadex G-100 Superfine column, one essentially symmetrical activity peak was obtained. Similar treatment of submandibular saliva also produced a single enzyme peak. Parotid saliva enzyme tolerated the purification procedures well: The specific activity before chromatography ranged from 8.7 to 16.0 U/mg. After chromatography the respective values were from 10.8 to 29.4 U/mg. However, the submandibular saliva enzyme decreased more than one half in specific activity under these conditions: Before chromatography 8.3–10.5 U/mg and after: 3.3–4.3 U/mg. All attempts to purify the enzyme further failed. Recovery of activity following molecular-permeation chromatography ranged between 10 and 20 % and between 5 and 10 % of that found in submandibular saliva and parotid saliva, respectively. In this study no attempts to restore the enzyme activity by chemical methods were made.

Isoelectric focusing of *M. mulatta* parotid saliva in a pH gradient of 7 to 9 resulted in three peaks with peroxidase activity. The isoelectric points and the specific activities of the enzyme peaks were: I, 6.1 (0.11 U/mg); II, 7.3 (0.10 U/mg); III, 8.4 (0.48 U/mg) (Fig. 1).

The lactoperoxidase of *M. fascicularis* parotid saliva was partially purified using the same procedures as described above. The enzyme had an isoelectric point of 7.9 in a pH gradient of 3 to 10. Submandibular saliva from this species was not studied.

Results of molecular-permeation chromatography on a Sephadex G-100 Superfine column indicated a molecular weight of 79 000 for both *M. mulatta* and *M. fascicularis* parotid saliva lactoperoxidase. The corresponding value for the submandibular saliva enzyme, determined for *M. mulatta* only, was 123 000. It should be noted, however, that these molecular weight values are only tentative inasmuch as the physicochemical homogeneity of these enzymes

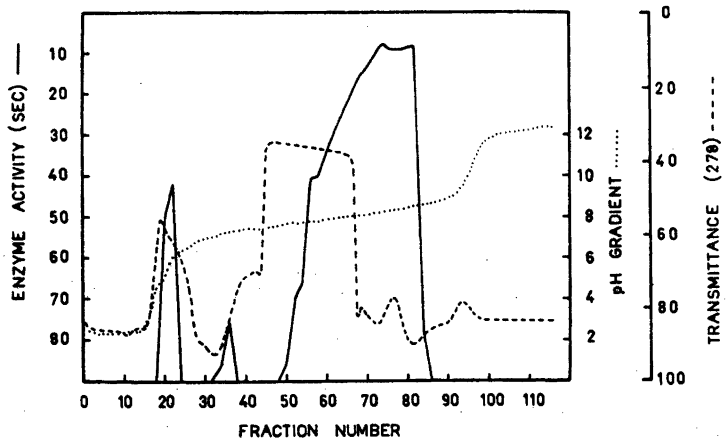

Fig. 1. Isoelectric focusing of *M. mulatta* parotid saliva lactoperoxidase in a pH gradient from 7 to 9. Voltage 600 V; current, < 10 mA; time of focusing, 96 h; temperature, 0 °C; sample, 8 ml (96 mg protein) of parotid saliva; fraction volume, 1.2 ml.

under the above experimental conditions has not as yet been ascertained.

The lacrimal fluid of *M. fascicularis* contained a lactoperoxidase which behaved upon CM-cellulose chromatography in a manner similar to that of the salivary enzymes. The specific peroxidase activity of lacrimal fluid was 4.8 U/mg protein before chromatography and 1.4 U/mg after it. All fractionation experiments described above were carried out twice. The results were essentially similar in both cases.

The kinetic experiments were carried out with the *M. fascicularis* parotid saliva lactoperoxidase after CM-cellulose chromatography, as this fraction had sufficient activity (68 U/mg) to carry out all experiments described. Furthermore, this enzyme gave in focusing after CM-cellulose chromatography only one enzyme peak. Hanes's plot gave a value of 1.5 mM for the substrate constant, K_s, in the oxidation of guaiacol. The value of K_s for H_2O_2 was determined at guaiacol concentration (5.0 mM) which produced maximum velocity. The plot of Lineweaver and Burk (Fig. 2) yielded a K_s value for guaiacol of 8.3 mM. The difference between the above values obtained from separate plots and experiments most likely resulted from small differences in the employed final concentrations of H_2O_2. For hydrogen peroxide the corresponding K_s value was 0.026 mM.

Potassium cyanide inhibited the lactoperoxidase-catalyzed oxidation of guaiacol. The inhibition was noncompetitive up to $[CN^-] = 0.5$ mM in the presence of 1 mM H_2O_2 (Fig. 2). Other plots * (v_i/v_o *vs.* $[CN^-]$, and the Dixon's plot of $1/v_i$ *vs.* $[CN^-]$ suggested that guaiacol (0.01 to 0.05 M) and CN^- ions (up to 0.5 mM) did not compete for the same binding site on the enzyme surface. The Dixon's plot gave a value of 0.25 mM for the inhibition constant, K_i, while the double reciprocal plot (Fig. 2) gave values ranging from 0.10 mM to 0.14 mM.

Maximal activity of the enzyme with 0.005 M guaiacol as substrate occurred between pH values 8 and 9. Clearly measurable activity was still observed at pH values 6.5 and 10.0. For the parotid saliva enzyme of *M. mulatta* a

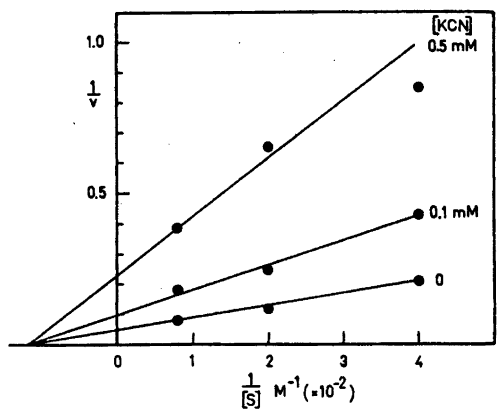

Fig. 2. The double reciprocal plot of the oxidation of guaiacol catalyzed by *M. fascicularis* parotid saliva lactoperoxidase, inhibited by CN^- ions. The reaction mixtures contained 0.4 ml of 0.01 M phosphate buffer (pH 7.0), 0.25 ml of 0.02 M guaiacol (*o*-methoxyphenol), 0.1 ml of KCN at various concentrations, and 0.25 ml of enzyme solution. The reactions were performed at +30 °C and started by adding 10 µl of 0.01 M H_2O_2 to the mixture.

* v_i, rate of reaction in the presence of inhibitor; v_o, rate of uninhibited reaction.

value of 2.6 mM was obtained for the K_s in the oxidation of guaiacol. This reaction was inhibited noncompetitively by CN^- ions with a K_i value of 0.1 mM. The effect of pH on this enzyme was similar to that described for the *M. fascicularis* enzyme.

This report suggests that the human and monkey salivary lactoperoxidases have some common properties such as sensitivity to CN^- ions and molecular weight values. The reported values of the molecular weight of human salivary peroxidase range from 73 500 to 89 000.[9,11] Values of 80 000 to 100 000 have been reported for the molecular weight of a human saliva antibacterial factor which most likely corresponded to lactoperoxidase.[12] Other similarities between the human and monkey parotid saliva lactoperoxidases are the isoelectric points of the main molecular forms.[10] The monkey salivary lactoperoxidases clearly differed from the human salivary enzyme in their lower stability upon being subjected to molecular-permeation chromatography and other purification steps after CM-cellulose fractionation.

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A Note on the Methyl Inductive Effect in Sulfides

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The methyl group is usually classified as electron-donating relative to hydrogen. This is partly based on the observed effect of methyl substitution on acid and base strengths. There are, however, several experimental observations that cast doubt on these interpretations,[1] supported by molecular orbital calculations showing the methyl group to be electron-attracting (relative to hydrogen) in simple alcohols and amines.[2]

It is the purpose of this communication to show that, according to molecular orbital theory, a methyl group is *electron-attracting* also when bound to sulfur in the forms commonly occurring in organic chemistry. Furthermore, we will show that the simple correlation that usually is assumed to exist between high charge density of an anion and a corresponding high proton affinity or gas-phase base strength, does not seem to be valid.

The quantum mechanical method used was the MO-LCAO-SFC method using a double-zeta Gaussian basis including 3*d*-orbitals on sulfur. Further computational details can be found elsewhere.[3] The molecular geometries used were partially optimized and partially based on previous experience in sulfur systems.[3,4]

The molecules studied were those listed in Table 1 together with the corresponding calculated proton affinities and atomic charges on sulfur. Also listed are the dipole moments of the neutral molecules as these will reflect redistribution of the electrons. The relation

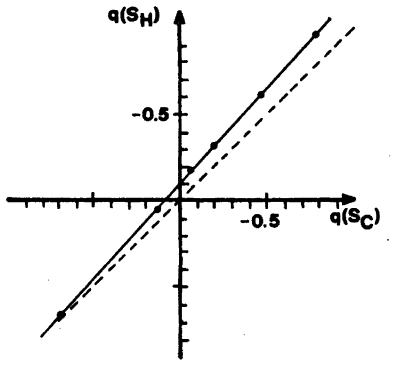

Fig. 1. The atomic charge on S bound to H, $q(S_H)$, *versus* the atomic charge on S bound to C, $q(S_C)$. The broken line represents equal charge on S_H and S_C.