SIMULTANEOUS RELEASE OF AMYLASE AND PEROXIDASE FROM THE GUINEA PIG SUBMANDIBULAR GLAND

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1. Introduction

It has long been known that the enzyme α-amylase is a component of salivary gland secretion products. Salivary glands have also been shown to have peroxidase activity [1,2]. The peroxidase present in the submandibular gland of the cow and the pig has been purified and characterized as a lactoperoxidase [3,4]. The biological significance of this enzyme and its role in salivary gland metabolism is, however, unclear [5]. Recent cytochemical investigations have shown not only that the secretory cells of certain salivary glands display a positive histochemical peroxidase reaction, but that a similar reaction is also observed in the excreted products of the glands [6–8]. The findings suggest that the enzyme is synthesized within salivary gland cells and is secreted into the duct system of the glands. The precense of peroxidase activity in the saliva has been established with biochemical assays [9]. The intracellular relationship between amylase and peroxidase is essentially unknown. Furthermore, although numerous reports in the literature have been concerned with the mechanisms underlying the synthesis and release of amylase, little is known of the factors governing the discharge of peroxidase from salivary glands. It was therefore considered of interest to study and compare the release of peroxidase and amylase in an in vitro system employing certain specific stimulants of secretion. The submandibular gland of the guinea pig is known to contain large amounts of both peroxidase [2] and amylase [10] and was therefore chosen for this study. Secretion of both amylase and peroxidase from submandibular slices was stimulated by adrenalin, isoprenalin and carbamylcholine. Adrenalin was a more potent stimulant of enzyme release than the other agents. The enzyme release caused by this catecholamine could be inhibited by 2,4-dinitrophenol, indicating that there is active secretion of both amylase and peroxidase.

2. Materials and Methods

Three-month-old male guinea pigs weighing roughly 300 g were used for the investigation. The animals were starved for 12 hr prior to sacrifice. They were anesthetized by an intra-peritoneal injection of sodium pentobarbital (Nembutal®, Abbott). The submandibular glands of both sides were removed. The glands were cut into small fragments, which were randomly distributed in the incubation vessels. All specimens were preincubated for 15 min in 3 ml of bicarbonate buffer (pH 7.4) supplemented with pyruvate, glutamate and fumarate [11]. After this preincubation the medium was removed and rapidly replaced by 3 ml of fresh incubation medium to which were added different stimulants or inhibitors of secretion. The compounds used were adrenalin, isoprenalin, carbamylcholine and 2,4-dinitrophenol

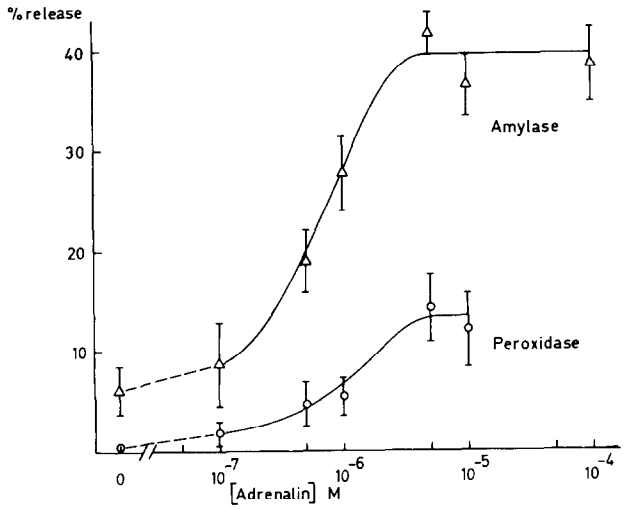

Fig. 1. Dose-response curves for release of amylase and peroxidase by adrenalin. The values are given ± S.E.M.

(DNP). After 30 min of incubation fresh adrenalin and isoprenalin were added to the media, because of the rapid autooxidation of these substances. Both preincubations and incubation were carried out at 37° under continuous gassing with 95% O_2 and 5% CO_2 in a shaking water bath. After 60 min of incubation the specimens were removed, weighed and homogenized in 3 ml of 50 mM phosphate buffer (pH 6.9) [12]. Amylase and peroxidase activities in media and homogenates were determined. Activities of the homogenates were determined on the supernatants after centrifugation at about 3000 g for 5 min.

2.1. *Peroxidase assays:*

Samples from the homogenates and incubation media were placed in 17 mM guaiacol at pH 7.0 (50 mM sodium phosphate). 0.3 mM hydrogen peroxide was added and the peroxidase activity was calculated from the increase in absorbance at 470 nm. The peroxidase activity added in the samples was kept within the limits of linearity of the method. The results were corrected, when necessary, for the influences of entrained incubation medium and stimulants or inhibitors. The enzyme activity was essentially independent of hydrogen peroxide concentration around 0.3 mM in 17 mM guaiacol and, as short reaction times were used, the catalase activity in the samples did not affect the peroxidase assay. The activities were expressed as ΔA_{470}/ wet weight per minute.

2.2. *Amylase assays:*

A modification to the micro scale of the 3,5-dini trosalicylic acid technique [12] was employed. 0.2 of sobule starch and 0.004 g of NaCl were dissoved in 10 ml 0.05 M phosphate buffer [12] (pH 6.9) an freshly prepared for each series of determinations. The sample to be analyzed was diluted 50–200 times with phosphate buffer in order ro suit the lim of the standard curve. 10 μl of the diluted sample w incubated with 10 μl of the starch solution for 10 n at 25°. The reaction was interrupted by adding 20 μl of the 3,5-dinitrosalicylate reagent. Zero-time control determinations were run for all samples. However, in this case the 3,5-dinitrosalicylate reage was added before the starch solution. After incubati all tubes were placed in boiling water for 10 min. Al cooling, 200 μl of distilled water was added. The co lour which developed was measured in a spectropho tometer at 530 nm. Blanks and standards containin maltose monohydrate in the concentrations 0, 1.0, 2.0 and 3.0 mg/ml were used in each analysis, and handled as described for the test samples. One unit of amylase was defined as the activity liberating reducing groups corresponding to 1 micromole of maltose monohydrate per minute. The amylase activ ity was related to g weight of the tissue.

3. Results

Tables 1 and 2 illustrate the effects of adrenalin, isoprenalin, carbamylcholine, DNP and adrenalin + DNP on the secretion of amylase and peroxidase fro the guinea pig submandibular gland. Of the compou tested, adrenalin was the most active secretagogue. DNP inhibited the adrenalin-stimulated secretion of both enzymes. The release of peroxidase in the abse of added stimulants was not appreciably affected by DNP, whereas DNP caused a slight inhibitation of the basal secretion of amylase. Dose-response curves for the effect of adrenalin on amylase and peroxidas release are shown in Fig. 1. Maximal secretion of both amylase and peroxidase was obtained at a concentration of 5×10^{-6} M. Concentrations of adrenal higher than 10^{-5} M gave low yields of peroxidase because of interference with the assay technique an

Table 1
In vitro release of amylase from the guinea pig submandibular gland.

	n	Amylase activity in medium (units/g wet weight, ± S.E.M.)	Amylase activity in hemogenate (units/g wet weight, ± S.E.M.)	Amylase released (% ± S.E.M.)
Control	11	310 ± 33	2433 ± 251	11.5 ± 1.0
DNP 10^{-3}M	5	107 ± 27	2206 ± 571	6.5 ± 2.0
Adrenalin 10^{-5}M	11	1128 ± 145	2125 ± 341	36.4 ± 3.1
Adrenalin 10^{-5}M + DNP 10^{-3}M	6	350 ± 64	3224 ± 323	10.3 ± 1.6
Carbamylcholine 5×10^{-5}M	5	650 ± 49	1607 ± 284	30.6 ± 3.9
Isoprenalin 10^{-4}M	6	1038 ± 185	2640 ± 450	29.1 ± 3.1

Table 2
In vitro release of peroxidase from the guinea pig submandibular gland.

	n	Amount of peroxidase in medium (ΔA_{470}/g wet weight per min ± S.E.M.)	Amount of peroxidase in homogenate (ΔA_{470}/g wet weight per min ± S.E.M.)	Peroxidase released (% ± S.E.M.)
Control	11	0.41 ± 0.08	36.2 ± 3.2	1.2 ± 0.2
DNP 10^{-3}M	5	0.4 ± 0.1	37.5 ± 5.3	1.2 ± 0.7
Adrenalin 10^{-5}M	11	6.9 ± 0.5	33.4 ± 3.1	17.8 ± 1.4
Adrenalin 10^{-5}M + DNP 10^{-3}M	6	0.3 ± 0.1	47.5 ± 5.6	0.8 ± 0.2
Carbamylcholine 5×10^{-5}M	5	2.3 ± 0.4	35.1 ± 5.8	6.3 ± 1.0
Isoprenalin 10^{-4}M	6	4.6 ± 0.5	45.4 ± 3.5	9.5 ± 1.5

probably also because of inactivication by the rapidly autooxidizing adrenalin.

4. Discussion

Exportable proteins, e.g. amylase, present in exocrine glands accumulate within membrane-bounded zymogen granules in the secretory cells, and these stores of granule-bound enzymes are rapidly diminishe during secretion [13]. In the guinea pig submandibula gland amylase activity has been found to be mainly localized to the acinar cells [10]. These cells also contain secretory granules which display a positive histochemical reaction for peroxidase at both the light and electron microscopic levels [8,14]. In other words, both peroxidase and amylase are present in secretory granules of the guinea pig submandibular gland, although it is not known to what extent the two enzymes may coexist in the same individual granule. Cholinergic agents, as well as adrenalin and noradrenalin have been shown to stimulate amylase secretion from salivary gland slices in vitro (e.g. [15,16]). According to the authors cited the catecholamines are more potent inducers of enzyme secretion from rat parotid gland slices than is carbamylcholine. These findings are in accord with the present observations. Adrenalin caused a release of 35–40% of the amylase of the guinea pig submandibular gland, whereas the secretion induced by carbamylcholine was somewhat less. Likewise, adrenalin was definitely a more potent secretagogue than carbamylcholine with respect to the release of peroxidase. Combined quantitative enzyme analyses and electron microscopic observations have shown that within 2 hr after a single injection of isoprenalin more than 90% of the amylase stored in the rat parotid gland is secreted, a phenomenon which is paralleled by a complete discharge of zymogen granules from the secretory cells [17]. In the present *in vitro* investigation isoprenalin was found to stimulate the release of both amylase and peroxidase from submandibular slices. At the concentration employed (10^{-4}M) its secretion-provoking effect was less than that of adrenalin, and similar to that of carbamylcholine. DNP, an inhibitor of oxidative phosphorylation, was found to abolish the adrenalin-induced secretion of both enzymes. This finding suggests that sustained release of amylase and peroxidase requires the continu

ous provision of ATP through the respiratory chain. The release of amylase and peroxidase into media without added stimulants amounted to about 10% and 1%, respectivily. This basal release of amylase was only slightly inhibited by DNP, and the basal release of peroxidase was not appreciably affected by this metabolic inhibitor. It seems therefore possible that small amounts of amylase and peroxidase may have passively leaked out from the slices during incubation. Whereas the function of amylase is well established, very little is known about the physiological role of peroxidase. The present results, together with previous studies on the localization of peroxidase in salivary glands and in saliva, clearly indicate that the enzyme originates in the salivary gland secretory cells and is discharged from the latter. During recent years it has been reported that lactoperoxidase, the peroxidase of salivary glands, together with thiocyanate and hydrogen peroxide is very effective in inhibiting the growth of certain bacteria [18], and that this effect is potentioted by halides, especially the iodide ion [19]. It is striking that peroxidase activity and ability to concentrate iodide from the blood occur in parallel in most salivary glands from eight examined species [20]. Furthermore, lactoperoxidase exerts a strong viricidal and fungicidal action [12,22]. According to [23] an antibacterial peroxidase system may well exist in the saliva. A similar function has also been attributed to the peroxidase present in leucocytes and in the Kupffer cells of the liver, cells which are well-known participants in the defence system of the body [24,25]. It has been suggested that the peroxidase present in the secretion products of the lacrimal gland acts in a similar way [26]. Practically all enzymes secreted from exocrine glands are hydrolases. From the present findings, however, it can be concluded that the oxidative enzyme peroxidase is secreted from salivary gland slices and responds to secretory inducers *in vitro*. Moreover, there was a striking similarity between the amylase and peroxidase secretory responses to the various stimulants and inhibitors tested. This result indicates that the mechanisms for control of peroxidase release are very similar to those regulating the release of amylase. Such a conclusion is in agreement with the previous electron microscopic data, which suggest that the two enzymes are similary stored and may even coexist in the same secretory granules. The present technique for studying the simultaneous release of amylase and peroxidase in vitro may be useful for the further biochemical exploration of this possibility.

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