RAT PLASMA CLEARANCE OF HORSERADISH PEROXIDASE AND YEAST INVERTASE IS MEDIATED BY SPECIFIC RECOGNITION

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1. Introduction

The extracellular survival of various native and modified glycoproteins is determined by the nature of the exposed sugar residues associated with the carbohydrate chains. The work [1] has provided the conceptual basis for selective hepatic recognition and uptake of modified glycoproteins. Exposure of the penultimate sugar (viz. galactose) on most mammalian plasma glycoproteins (e.g. by treatment with neuraminidase) results in a marked increase in their respective plasma clearance rates. A liver hepatocyte plasma-membrane 'lectin' which mediates binding of galactose-terminating glycoproteins has been isolated and characterized [2]. More recently, evidence for a second clearance pathway has emerged [3–6]. The latter is distinct from the galactose system in that it is localized predominantly in liver Kupffer cells and mediates recognition and uptake of glycoproteins with mannose/*N*-acetylglucosamine in the terminal position [7–9]. Several lines of evidence suggest that mannose and *N*-acetylglucosamine terminal glycoproteins are recognized by the same receptor system. A study [10] showed that mannose and *N*-acetylglucosamine terminal glycoproteins compete for clearance in vivo. More recently, binding studies with alveolar macrophages [11] have demonstrated cross-inhibition by glycoproteins terminating in mannose and *N*-acetylglucosamine when tested against each other. Of possible physiological importance is the observation that most lysosomal glycosidases are recognized by the mannose/*N*-acetylglucosamine system in vivo [7,12].

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In the present study, two widely used markers for non-specific endocytosis, horse-radish peroxidase (HRP) and invertase, have been shown to be cleared in vivo by specific recognition. Evidence is presented which indicates that sugar residues on the enzymes act as recognition determinants which mediate rapid plasma clearance.

2. Methods and materials

2.1. *Enzymes and assays*

Horseradish peroxidase (Type VI) was obtained from Sigma. Activity was assayed at 25°C by the diaminobenzidine method in [13]. Units are ΔA_{465}/min. Gel electrophoresis of the commercial product in the presence of sodium dodecyl sulfate [14] revealed one major band when stained for protein. Type VI HRP was separated into its component isozymes (A,B,C) by chromatography on CM-cellulose after the method in [15]. The specific activities of the components following chromatography were slightly elevated when compared to the original material (16.4×10^{-3} units/mg). The respective activities (10^{-3} units/mg) were: isoenzyme A, 19.5; isoenzyme B, 24.8; isoenzyme C, 23.5.

Invertase (Sigma, grade VII) was further purified by the method in [16] using DEAE-cellulose. Activity was assayed with sucrose as substrate by the method in [17]. Units at 37°C are μmol glucose produced/min. The specific activity was enhanced about 12-fold (79–934 units/mg) by the DEAE-cellulose step. The sugar composition of the purified invertase was estimated at 46% (mg/mg protein) by the phenol–sulfuric acid test [18]. SDS–gel electrophoresis of purified

material demonstrated a single major protein band. β-Glucuronidase was purified from rat preputial glands as in [12]. The purified material was homogeneous by gel electrophoresis and had a specific activity of >2000 units/mg using phenolphthalein glucuronide as substrate. Assay of β-glucuronidase in blood plasma was made with 4-methylumbelliferyl-glucuronide as the substrate [19]. Yeast mannan was from Sigma. Protein was measured by the method in [20] using bovine serum albumin as the standard. Monodisperse carboxylated monodisperse microspheres (0.278 μm diameter) were purchased from Polysciences, Inc. Colloidal silver (Neosilvol) was a gift from Parke Davis Co.

2.2. *Clearance studies*

Clearance of enzyme was followed in the anesthetized female Wistar rat (Harlan Industries, Cumberland, IN) as in [12]. Briefly, cannula were placed into the femoral vein and artery. Enzyme samples were infused intravenously in total vol. 0.5 ml 0.15 M NaCl using a Harvard constant-infusion pump. Delivery of the enzyme continued over a 70 s period. The first blood samples was taken at 90 s and at the indicated times thereafter. Heparinized hematocrit tubes were used for sampling arterial blood. Enzyme assays were performed on plasma after separation in a hematocrit centrifuge. Inhibitors were added directly to the infused enzyme samples to achieve simultaneous delivery of the two.

2.3. *Periodate oxidation*

HRP (A) (1 mg/ml) was incubated in 0.05 M sodium acetate, pH 5.0, containing 0.01 M sodium metaperiodate at 4°C in the dark. The reaction was stopped after 60 min by the addition of 0.1 M ethylene glycol followed by dialysis against 5 mM phosphate, pH 6.5. The recovery of enzymatic activity was 42%.

Invertase (1.5 mg/ml) was incubated as above except the reaction was terminated at 7 h. The recovery of invertase activity was 55%.

3. Results

3.1. *Effect of yeast mannan on plasma survival of horseradish peroxidase and yeast invertase*

The fact that both HRP and invertase are mannose-rich glycoproteins [15,16] suggested to us that clearance may be mediated by the same system which mediates plasma clearance of lysosomal glycosidases [4].

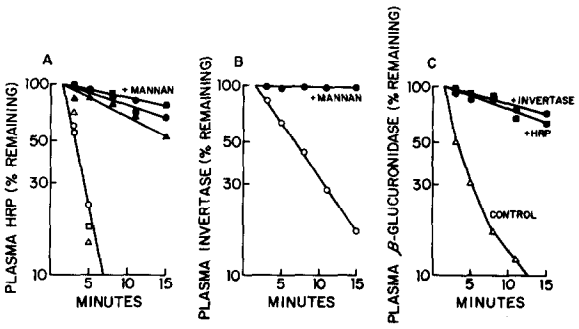

Fig.1A. Plasma clearance of 1000 units HRP isozymes A (○), B (△) and C (□) in the absence (open symbols) and presence (closed symbols) of 5 mg yeast mannan. 1B. Clearance of 20 units invertase in absence (○) and presence (●) of 5 mg mannan. 1C. Clearance of 10 units β-glucuronidase in the absence (△) and presence of 2.3 mg HRP (■) or 5 mg invertase (●). Plasma levels of enzyme are expressed as % first post-infusion plasma sample.

Tracer (test) doses of HRP and invertase, in separate experiments, were infused intravenously into the anesthetized rat. Assay of plasma for enzyme activity following termination of the infusion indicated that both HRP and invertase (fig.1B) were rapidly cleared with half-survival times of < 5 min and < 10 min, respectively. HRP which had been further fractionated into its component isozymic forms, A, B and C by ion-exchange chromatography, was tested for rapid clearance. All three forms of HRP were rapidly cleared from plasma. The clearance profiles of the three preparations were indistinguishable (fig.1A).

Manna is one of the most potent inhibitors of lysosomal glycosidase clearance in vivo [7,8]. To test for inhibition of HRP and invertase clearance by mannan, animals were infused as before with tracer doses of HRP and invertase, respectively, along with 5 mg yeast mannan. The results in fig.1 show that clearance of all three forms of HRP as well as invertase, was severly impaired by the presence of mannan.

3.2. *Effect of HRP and invertase on the clearance of rat β-glucuronidase*

Lysosomal glycosidases are rapidly cleared in vivo

where clearance is inhibited by mannose- and/or *N*-acetylglucosamine-terminal glycoproteins. A cross-inhibition experiment, using HRP and invertase as antagonists and rat β-glucuronidase as agonist, was undertaken to show that recognition and clearance of all three enzymes are mediated by the same system. As shown [4,12,19] rat preputial gland β-glucuronidase is rapidly cleared in vivo (fig.1C). When administered with a blocking dose of HRP or invertase, clearance of β-glucuronidase is severely impaired (fig.1C).

3.3. *Effect of periodate oxidation, colloidal silver and latex beads on clearance of HRP and invertase*

Periodate oxidation virtually abolishes the recognition and clearance of lysosomal glycosidases in vivo [19]. The results of the previous experiment support the concept that sugar residues associated with HRP and invertase are essential for recognition in vivo. To test the effect of periodate oxidation, HRP and invertase were incubated with sodium periodate as described in section 2. Results from clearance experiments using periodate-oxidized enzyme are summarized in table 1. Periodate oxidation of HRP and invertase completely abolished their recognition in vivo and produced an enzyme which has a very long plasma half-survival time.

To rule out the possibility that non-specific uptake by liver could account for in vivo clearance, animals were injected with latex beads or colloidal silver simultaneously with a test dose of HRP or invertase. Neither beads nor colloidal silver had an effect on enzyme clearance. The dose of colloidal silver used had been demonstrated in earlier experiments effectively to block the clearance of ^{198}Au-colloid [12].

4. Discussion

This report summarizes studies on the extracellular survival of two glycoprotein enzymes, yeast invertase and horseradish peroxidase. The study was undertaken because both enzymes are mannose-rich glycoproteins which have been extensively employed as markers for non-specific uptake by cells and tissues. The results confirm earlier reports that HRP [21] and invertase [22] are rapidly cleared in vivo. The experiments indicate that uptake of both markers by cells in vivo is highly specific. First, clearance of HRP or invertase is unaffected by doses of colloidal material which saturate non-specific clearance by reticuloendothelial cells. Second, periodate oxidation of HRP or invertase abolishes their rapid clearance. Periodate oxidation had been shown previously to abolish the rapid clearance of lysosomal β-glucuronidase and *N*-acetyl β-D-glucosaminidase without appreciably

Table 1
Effect of periodate oxidation, colloidal silver and latex beads on clearance of horseradish peroxidase and invertase

Enzyme	Treatment	% Remaining in plasma	
		5 min	30 min
HRP (A) (1000 units)	Control	20	<1
	Colloidal silver	34	<1
	Latex beads	35	<1
	Periodate oxidation	98	96
Invertase (20 units)	Control	64	<4
	Colloidal silver	63	<2
	Latex beads	65	<3
	Periodate oxidation	89	89

HRP and invertase were prepared, assayed and oxidized with sodium periodate as described in section 2. % Remaining = (units/ml at time x ÷ units/ml in first plasma sample) × 100. Colloidal silver (25 mg) (shown in earlier studies to be an effect antagonist to the clearance of ^{198}Au-colloid [12]) and 0.4 ml microspheres (2.5% solids; 0.28 μm diameter) were administered simultaneously with enzyme

affecting enzymatic activity [19]. The uptake of HRP by neurons has also been shown to be periodate-sensitive, suggesting that neurons specifically recognize HRP and that an intact carbohydrate structure is important in the recognition process [23]. Third, the clearance of HRP and invertase is blocked by mannan. Conversely, HRP and invertase are effective antagonists to the clearance of β-glucuronidase. β-Glucuronidase and other lysosomal glycosidases have been shown to be cleared in vivo [7,19] and bound in vitro [11] by a clearance system that requires a terminal non-reducing mannose [7,8] or *N*-acetyl-glucosamine residue [4]. The results presented here are consistent with the view that HRP and invertase are cleared by the same mechanism which mediates recognition and clearance of lysosomal glycosidases. These conclusions are also consistent with the carbohydrate composition of HRP [15] and invertase [16] having a substantial mannose content, although exact sugar sequences have not been resolved. This study highlights the importance of exercising caution when employing HRP or invertase as markers for non-specific uptake in vivo.

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