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Iodination of α -Amylase using Solid State Lactoperoxidase¹⁾

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A radioiodination method using bovine lactoperoxidase covalently coupled with Sepharose-4 B, was successfully applied to α -amylase. Unlike some chemical iodination procedures, the lactoperoxidase method appeared to be quite gentle, employing only 5–10 μ Ci Na^{125}I and traces of hydrogen peroxide as oxidising agent. The catalytic activity of the labelled enzyme appeared to depend on the total iodide to amylase molar ratio. Ratios greater than 0.6 reduced the catalytic activity. There was no loss of enzyme activity when iodinated at ratios up to 0.6. Amylase labelled under these conditions appeared suitable for metabolic studies. When canine plasma amylase concentration was artificially raised (5–10-fold), the disappearance curves of radioactivity and amylase activity were parallel. Tracer studies in dogs are also described.

Unter Verwendung einer an Sepharose 4B kovalent gebundenen Rinder-Lactoperoxidase ist es gelungen, Amylase radioaktiv zu jodieren. Im Vergleich zum chemischen Verfahren verläuft die Lactoperoxidase Methode unter schonenden Bedingungen. Lediglich 5–10 μ Ci Na^{125}I und Spuren von Wasserstoffperoxid als Oxidationsmittel werden benötigt. Die katalytische Aktivität des markierten Enzyms scheint von dem molaren Verhältnis des gesamten Jodids zur Amylase abhängig zu sein. Ein Verhältnis größer als 0,6 vermindert die katalytische Aktivität. Bei einem molaren Verhältnis unter 0,6 trat kein Aktivitätsverlust ein. Auf diese Weise markierte Amylase scheint für Stoffwechseluntersuchungen geeignet zu sein. Nach experimenteller Erhöhung der Konzentration der Plasma-Amylase bei Hunden auf den 5–10fachen Normalwert verliefen die Abnahmekurven von Radioaktivität und Amylase-Aktivität parallel. Tracer-Untersuchungen an Hunden werden beschrieben.

There are few reports in the literature on the successful labelling of enzymes without loss of catalytic activity. In the case of α -amylase (EC 3.2.1.1) the iodine monochloride method (1) caused severe loss of enzyme activity (2, 3). The method of Greenwood et al. (4) has been used for many enzymes (5–9). Chloramine-T can cause oxidation of amino acids other than tyrosine resulting in protein denaturation (10) and loss of catalytic activity (6). These disadvantages gave impetus to the search for a more gentle means of radioiodination. The electrolytic method (11) is reported to be very mild. It was used for α -amylase (3, 12) with minimal loss of enzyme activity.

Recently there have been several reports of successful iodination of proteins by means of the enzyme lactoperoxidase (EC 1.11.1.7) (10, 13, 14). It was used by these workers in soluble form in trace labelling albumin and immunoglobulins. Soluble lactoperoxidase has also been used in labelling proteins on the outer surfaces of mammalian cells without loss of cell viability (13, 15, 16). The method can also give high specific activities suitable for radioimmunoassay (17, 18).

An improved method employing solid state lactoperoxidase has been described (19, 20). By covalently coupling the enzyme to an insoluble matrix such as Sepharose-4B

it can be removed from the iodination reaction mixture simply by centrifugation. Moreover, very low concentrations of oxidising agent i.e. hydrogen peroxide and radioactivity are required. For these reasons, the method was studied for the iodination of α -amylase as described below.

Materials

Enzymes

Bovine lactoperoxidase and porcine α -amylase were purchased from Sigma Chemical Co., London, U.K. Canine amylase was purified from canine pancreatic juice (1000 U/mg protein).

Chemicals

All reagents were of Analar grade except the following which were obtained from the sources indicated: bovine albumin (fraction V), sodium azide and glycine, Sigma Chemical Co., London, U. K.; PHADEBAS blue starch tablets, blue dextran and cyanogen bromide activated Sepharose 4B, Pharmacia, London, U.K.; Bio-Gel P100, (50–150 mesh), Bio-Rad Laboratories, Richmond, California; hydrogen peroxide and sodium metabisulphite, Hopkin & Williams, Chadwell Heath, U.K.; Na^{125}I , Amersham, U.K.; human γ -globulin, Mann Research

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Laboratories, New York; *L*-cysteine hydrochloride, British Drug Houses, Poole, U.K.; ^{131}I -labelled albumin, Ames Company, Miles Laboratories, Slough, Buckinghamshire, U.K.; chloramine-T, May & Baker, Dagenham, U.K.

Procedures

Effect of chloramine-T on α -amylase action

75 μg porcine α -amylase was incubated with increasing quantities (2–80 μg) of chloramine-T in 50 μl 0.05 mol/l phosphate buffer containing 50 mmol/l NaCl and 0.5 mmol/l CaCl_2 . The incubation was for 1 minute at 22°C. 0.5 ml of sodium metabisulphite (1 mg/ml) was then added, the mixture dialysed and the catalytic activity of the enzyme determined after suitable dilution. Comparison was made with a control without chloramine-T pre-treatment, the value of which was taken as 100% activity. The effect of time on the reaction was studied as follows. 75 μg amylase was incubated with 80 μg chloramine-T as above. Aliquots of the reaction were removed at 10, 25, 35, 45 and 60 seconds and added to 0.5 ml of sodium metabisulphite prior to dialysis and analysis.

Preparation of solid-state lactoperoxidase

This procedure was performed as described by David (19). By recording the extinction at 412 nm before and after coupling, the enzyme was calculated to be coupled to the gel to a final concentration of 2.8 mg/ml of settled beads. The Sepharose bound lactoperoxidase was stored at pH 7.4 in 0.01 mol/l phosphate buffered saline in a volume such that 1.0 ml of suspension contained 1 mg of lactoperoxidase.

Iodinations:

The procedure of David (19) was followed. All iodinations were performed using the following concentrations of reactants unless otherwise indicated: lactoperoxidase, 10 $\mu\text{g}/\text{ml}$; potassium iodide, 10 $\mu\text{mol}/\text{l}$; hydrogen peroxide, 90 $\mu\text{mol}/\text{l}$; phosphate buffer, 0.05 mol/l containing 0.05 mol/l NaCl and 0.5 mmol/l CaCl_2 , pH 7.0; Na^{125}I containing about 10^7 c.p.m. (i.e. $\sim 5 \mu\text{Ci}$). Following iodination, and the removal of the Sepharose bound lactoperoxidase by centrifugation (3000 g, 5 min), the supernatant was dialysed in Visking tubing against several changes of phosphate buffer pH 7.0 until the counts in the external fluid disappeared. In some cases, i.e. when iodination reaction volumes of 0.1 ml or 0.2 ml were used and in the time curve experiments the reaction was stopped with 0.8 ml of 5 mmol/l cysteine (10) before centrifugation and dialysis.

The course of the iodination reaction was followed by precipitating aliquots of the reaction mixture, with trichloroacetic acid (19) at timed intervals up to 30 minutes. The radioactivity in the supernatant and precipitate was then counted in a Tracerlab gamma/guard 150 spectro/matic counter (ICN, U.K.). The percentage precipitability of the radioiodine in the supernatant after removal of the Sepharose bound lactoperoxidase was determined. Per cent loss of the ^{125}I counts due to "self-iodination" of the lactoperoxidase (at $t = 30$ min) was calculated from the total iodine remaining in the supernatant and precipitate as compared to the zero time sample. Per cent incorporation of counts into α -amylase was based on the total radioactivity added at zero time. ^{125}I utilization (at t_{30}) = % "self-iodination" + % ^{125}I incorporation (20). The free ^{125}I (%) of Table 1 (at t_{30}) = non utilized ^{125}I , i.e. 100– ^{125}I utilized.

Gel filtration

A Bio-Gel P-100 column (2 \times 62 cm) was equilibrated and eluted with 100 mmol/l NaCl containing 0.5 mmol/l CaCl_2 and 10 $\mu\text{mol}/\text{l}$ albumin. Aliquots of iodinated enzyme diluted to 1.0 ml with elution fluid were applied to the column, and fractions (2.25 ml) were collected at a flow rate 12–15 ml/hour.

Comparison of labelled and unlabelled amylase in substrate binding:

25 mU of labelled amylase was added to increasing amounts of blue starch substrate in 1.0 ml of phosphate buffered saline pH 7.0 at room temperature. After 2 minutes each tube was centrifuged at 2000 g (5 minutes) and the substrate washed

and centrifuged 5 times with 1.0 ml aliquots of the same buffer. The washed blue starch substrate and pooled supernatants were then counted and the bound enzyme (%) calculated. The substrate binding of unlabelled amylase (52 mU) was studied by measuring enzyme activity with increasing substrate concentration.

Experimental Animals

Three conscious female dogs (*Canis familiaris*) weighing 12–15 kg were used. Urine samples were obtained via catheter inserted into the bladder prior to the experiment. Blood sampling was facilitated by venous catheter in the leg or neck, fitted one day prior to the experiment. The animals were fed potassium iodide for two days beforehand in order to block the thyroid (21).

Comparison of the physiologic behaviour of radiolabelled and unlabelled amylase

The serum disappearance curves and renal clearances of ^{125}I -labelled and unlabelled canine amylase were determined in three dogs. Labelled amylase and sufficient cold amylase to raise the serum amylase level 5–10-fold was injected intravenously in 10 ml of physiological saline over a 1–2 minute period. Six heparinised blood samples were taken over the first hour and then at hourly intervals up to 6 hours. Each blood sample was replaced with a similar volume of physiological saline to maintain the blood volume constant. Urine was collected every 60 minutes over a 6 hour period. Each serum and urine sample was analysed for radioactivity, amylase activity and creatinine. Serum disappearance curves were constructed and renal clearance of amylase and radioactivity calculated (12) and expressed relative to the creatinine clearance.

Tracer studies

Serum disappearance curves and renal clearances were studied at normal serum amylase levels after injection of tracer quantities (15 U) i.e. $\sim 1\%$ of total serum amylase. Samples were collected as described above.

Assay methods

α -Amylase was measured by the method of Ceska et al. (22) and incorporating 7 $\mu\text{mol}/\text{l}$ albumin in the reaction mixture (23). Urine creatinine was measured by the method of Bonsnes & Taussky (24) and plasma creatinine by a modification of the same method (25). Haematocrits of all blood samples were measured. Blood volumes were measured by intravenous injection of ^{131}I -labelled albumin ($\sim 1 \mu\text{Ci}$) and measuring the degree of dilution using a Volemetron (Atomium, Billerica, Mass.) apparatus. This injection was administered with the α -amylase. The interference of these ^{131}I counts with the ^{125}I -labelled amylase counts was minimal and was calculated via a standard curve. An appropriate correction factor was then applied. Plasma volume was calculated from the blood volume and haematocrit values. The rate constants were calculated according to Matthews (26).

Results

Effect of chloramine-T on α -amylase activity

α -Amylase was found to be very sensitive to chloramine-T. The results of a 1 minute incubation of α -amylase with varying concentrations of chloramine-T is shown in Figure 1 a. The addition of 50 μg chloramine-T to 75 μg amylase/50 μl , reduced the enzyme activity to 39.3% of the control, while 80 μg almost abolished the catalytic activity. A time curve relating the effect of chloramine-T (80 μg) on α -amylase with time is shown in Figure 1 b. Within 10 seconds 60% of the enzyme activity was lost. This loss was progressive; over 90% of the catalytic activity was destroyed within 1 minute.

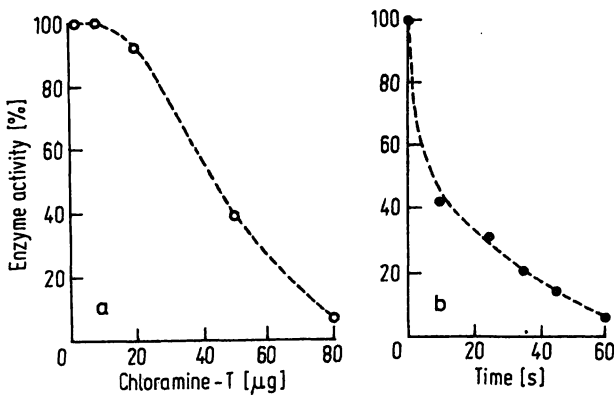


Fig. 1. (a) Effect of Chloramine-T (1 min, 22°C) on α -amylase activity (75 μ g enzyme protein/50 μ l).
 (b) Effect of Chloramine-T (80 μ g) on α -amylase activity as a function of time (75 μ g enzyme protein/50 μ l).

Iodination of α -amylase by solid state lactoperoxidase

The concentrations of lactoperoxidase, KI, H₂O₂ and buffer system used were those of David (19). The efficiency of iodination of α -amylase at a concentration of 1.3 mg/ml (Table 1) and the rate of iodine incorporation (Fig. 2) were similar to those obtained with IgG (19) at a comparable protein concentration. The effect of varying amylase concentration is also shown in Table 1. The fact that ¹²⁵I 'loss' due to self-iodination of Sepharoselactoperoxidase is lower at low amylase concentrations is surprising and differs from the findings of

Tab. 1. Iodination of α -amylase by lactoperoxidase at different amylase concentrations 10 μ g/ml lactoperoxidase; 10 μ mol/l KI; 5 μ Ci¹²⁵I; 90 μ mol/l H₂O₂; phosphate buffered saline, pH 7.0.

Amylase [mg/ml]	Cl ₃ CCOOH precipitability [%]	¹²⁵ I incorporation [%]	¹²⁵ I 'loss' [%]	¹²⁵ I free [%]	Total ¹²⁵ I utilization [%]
0.15	61.5	48	21.4	30.6	69.4
0.3	66	47.5	28.4	24.1	75.9
1.3	81.6	45	45	10	90

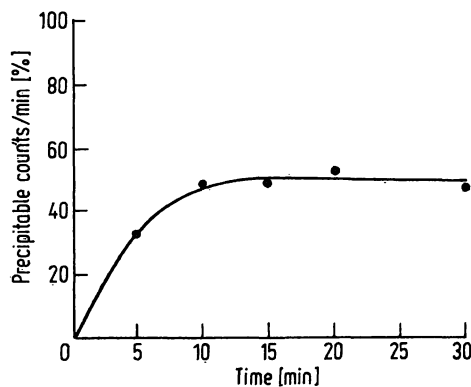


Fig. 2. Time curve showing ¹²⁵I incorporation into porcine α -amylase (0.3 mg/0.2 ml) by Sepharose-bound lactoperoxidase.

David (19). One would have expected that at high amylase concentrations less ¹²⁵I would be lost. Instead we see that at 0.15 mg amylase/ml, 21.4 % ¹²⁵I is 'lost' while at 1.3 mg/ml 45 % is 'lost'. The reason for this is not clear at present. However, the total ¹²⁵I lost i.e. due to self-iodination by sepharose lactoperoxidase plus free ¹²⁵I appears to be constant. This amounts to 52 % at 0.15 mg/ml and 55 % at 1.3 mg/ml (Table 1).

It is apparent from Table 2 that when the amylase concentration is kept high (i.e. by reducing the reaction volumes), ¹²⁵I incorporation decreases with decreasing mass of amylase. There is 45 % incorporation at 1.3 mg/ml with only 33.3 % at 0.15 mg/0.1 ml. This effect is not apparent at low amylase concentrations (Table 1). A corresponding reduction in % precipitability of iodinated amylase was found, 42.4 % when iodinated at 0.15 mg/0.1 ml (Table 2) compared with 61.5 % at 0.15 mg/ml (Table 1). The higher degree of ¹²⁵I incorporation at low amylase concentrations could be due to a structural change in the amylase molecule depending on the concentration. At low concentrations this conformational change may make more tyrosine residues accessible for iodination.

Effect of iodination on catalytic activity of α -amylase

Working with an amylase concentration of 1.3 mg/ml there appeared to be no loss in enzyme activity (Table 3).

Tab. 2. Iodination of α -amylase by sepharose-bound lactoperoxidase at constant amylase concentrations. 10 μ g/ml lactoperoxidase; 10 μ mol/l KI; 5 μ Ci¹²⁵I; 90 μ mol/l; phosphate buffered saline pH 7.0.

Amylase	Cl ₃ CCOOH precipitability [%]	¹²⁵ I incorporation [%]	¹²⁵ I 'loss' [%]	Total ¹²⁵ I utilization [%]
0.15 mg/0.1 ml	42.4	33.3	21.1	54.4
0.3 mg/0.2 ml	47.7	42	17	59
1.3 mg/ml	81.6	45	45	90

Tab. 3. Dependence of amylase activity on iodide/amylase molar ratio during iodination by lactoperoxidase. 10 μ g/ml lactoperoxidase; 10 μ mol/l KI; 90 μ mol/l H₂O₂; 5 μ Ci¹²⁵I; phosphate buffered saline, pH 7.0.

Amylase [mg]	Volume [ml]	Iodide/Amylase ratio	Enzyme Activity [%]	Radioactivity [counts/min · U]
0.15	1.0	3.3	32.5	
0.3	1.0	1.66	55	
0.65	1.0	0.8	75	
1.3	1.0	0.5	100	12,500
0.15	0.1	0.33	100	50,100
0.075	0.1	0.6	100	571,000*

* 50 μ Ci

However, iodination at a concentration of 0.15 mg/ml caused a marked reduction in enzyme activity to 32.5%. Even at 0.65 mg/ml enzyme activity was reduced to 75%.

The reason for this reduction in catalytic activity was then investigated. Initial experiments showed that the enzyme at a concentration of 0.15 mg/ml was not sensitive to 90 $\mu\text{mol/l}$ H_2O_2 on its own. Neither was it sensitive to the other components of the reaction, i.e. KI, ^{125}I , or Sepharose bound lactoperoxidase since a control experiment, without the H_2O_2 , had no effect on enzyme activity. The effect of temperature on the iodination was investigated since it was thought that iodination at 0°C might prevent denaturation at low amylase concentrations. It is shown in Table 4 that iodination at 0°C had a somewhat more adverse effect. The loss in catalytic activity was greater than when iodinated at room temperature. However, low temperature had no great effect on iodination efficiency as is evident from the figures for ^{125}I incorporation (Table 4).

The loss of enzyme activity appears to be a function of the iodide/amylase molar ratio (Table 3). In the case where 32.5% enzyme activity remains a ratio of 3.3 is present. At an iodide/amylase value of 0.8, enzyme activity is 75%, while at a ratio of 0.5 there is 100% catalytic activity. To iodinate 0.15 mg of amylase, therefore, without losing activity the enzyme concentration must be kept high relative to the iodide concentration. This is done by reducing the reaction volume containing 0.15 mg amylase to 0.1 ml. The iodide/amylase ratio is now reduced to 0.33 giving full enzymatic activity. Ratios ≥ 0.8 have a deteriorating effect. By varying the quantity of ^{125}I , high specific activities can be obtained, e.g. using 50 μCi ^{125}I and 0.075 mg amylase/0.1 ml a specific activity of 571,000 cpm/unit enzyme was obtained (Table 3). Such specific activities are desirable for tracer studies. α -Amylase, purified from canine pancreatic juice, behaved similarly to the porcine enzyme with regard to ^{125}I -incorporation, precipitability and sensitivity to high iodide/amylase ratios.

Gel filtration of labelled amylase

The behaviour of labelled amylase (iodinated at iodide/amylase ratio of 0.33) on a Bio-Gel P-100 gel filtration

Tab. 4. Effect of temperature on iodination reactions 10 $\mu\text{g/ml}$ lactoperoxidase; 10 $\mu\text{mol/l}$ KI; 90 $\mu\text{mol/l}$ H_2O_2 ; 5 μCi ^{125}I ; phosphate buffered saline, pH 7.0.

Amylase [mg/ml]	Temperature [$^\circ\text{C}$]	Cl_3CCOOH precipit- ability [%]	^{125}I incor- poration [%]	Enzyme Activity [%]
0.15	22	61.5	48	32.5
	0	68	46	20
0.3	22	66	47.5	55
	0	50.3	41.3	50

column is shown in Figure 3. Its elution profile coincided with enzyme activity. When iodinations were performed at high iodide/ amylase ratios causing catalytic activity to be reduced, the radioactive peak on gel filtration emerged slightly to the left of the main enzyme peak. This indicates that loss in activity is accompanied by a change in structure of the labelled molecules, which is detectable on gel filtration.

Comparison of labelled and unlabelled amylase in substrate binding

The ability of labelled amylase to combine with blue starch was studied as described in Procedures. Figure 4, curve (a) shows a plot of the % labelled enzyme bound to substrate vs. substrate concentration. A parallel curve (b) was obtained for unlabelled enzyme. The parallel curves indicate that the labelled product is combining with its substrate in a similar manner to the unlabelled enzyme.

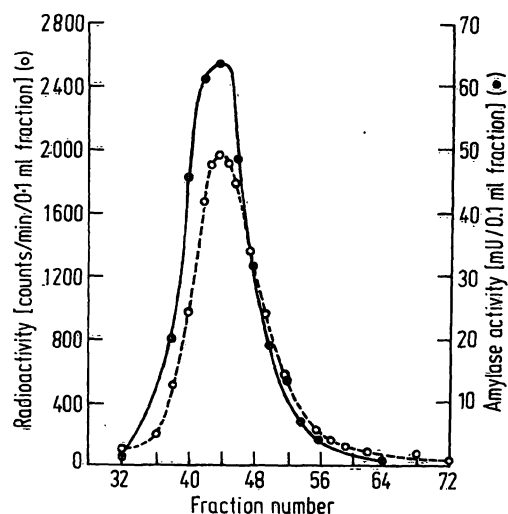


Fig. 3. Elution profile of ^{125}I -labelled amylase (o) and enzyme activity (e) on Bio-Gel P100 (V_t 157 ml; V_o 46 ml; fraction vol 2.25 ml; Eluent 0.1 mol/l NaCl, 0.5 mmol/l CaCl_2 and 10 $\mu\text{mol/l}$ albumin).

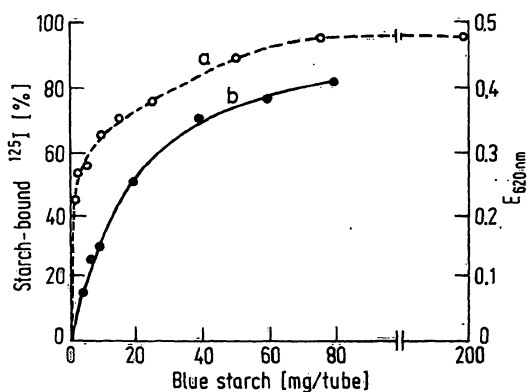


Fig. 4. Substrate-bound ^{125}I -labelled amylase vs. substrate concentration (a) compared with enzyme activity vs. substrate concentration (b).

Comparison of plasma disappearance curves of labelled and unlabelled amylase

The disappearance curves of radioactive amylase and amylase activity were compared in dogs after intravenous injection of a mixture of labelled and unlabelled enzyme as described in Procedures. In each of three studies, radioactivity disappeared from the plasma at almost the same rate as did amylase activity until normal serum amylase levels were approached. The curves could be resolved into at least two exponential components (26). The typical result is shown in Figure 5. This iodinated amylase appeared to trace amylase catabolism since the specific activity of the serum (cpm/amylase activity) remained constant over the 6 hours. If an appreciable difference existed in the rates of catabolism of the labelled and unlabelled enzyme, the specific activity would be expected to rise or fall above that injected. The fact that it remained constant is satisfactory since metabolic turnover studies require that the iodinated and non-iodinated molecules are catabolised and excreted at the same rate (21, 26).

Tracer studies

Tracer quantities of ^{125}I -labelled amylase were administered intravenously to two dogs. The typical result is shown in Figure 6 a. The % of dose remaining in the plasma is plotted against time and represented by line Q_p . Urine samples were also measured for radioactivity and from each figure the amount of label remaining in the body at that time could be calculated. The quantity of enzyme remaining was then plotted as a function of

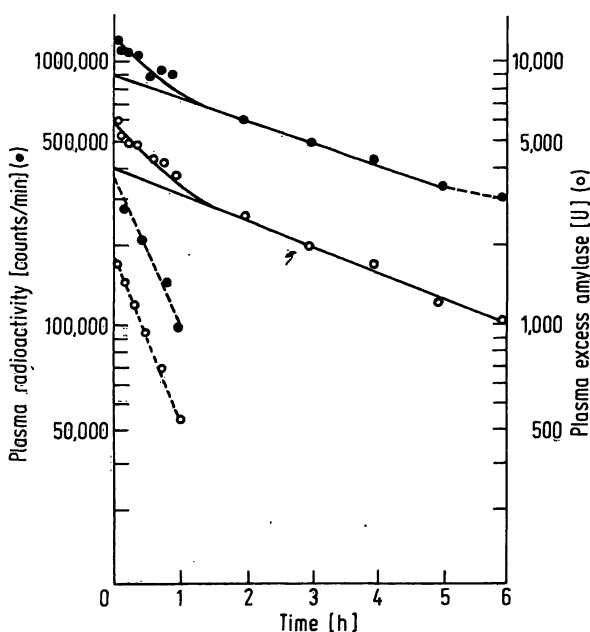


Fig. 5. Simultaneously measured plasma disappearance curves for amylase activity and radioactivity in the dog after i.v. injection of labelled and unlabelled enzyme. The solid line represents measured values, while the dotted line is computed by extrapolating the steady-state fall-off back to zero time and subtracting the resulting line from the observed data.

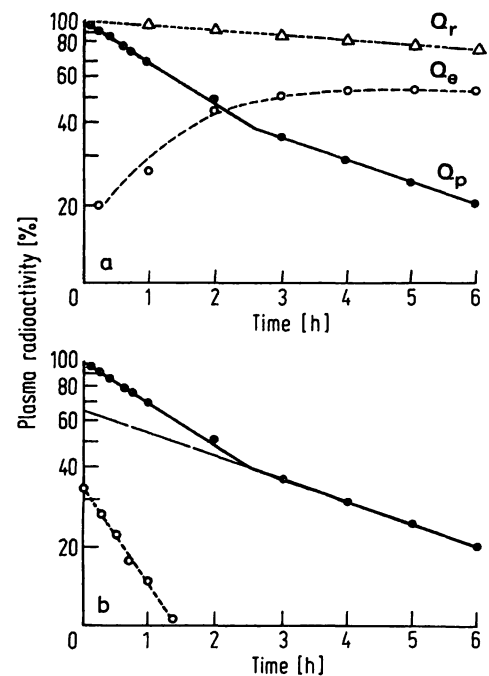


Fig. 6. (a) ^{125}I -labelled amylase turnover data Q_p = plasma curve, Q_r = total body curve i.e. total dose minus urine, Q_e = extravascular curve calculated from $Q_r - Q_p$. (b) Plasma curve (\bullet) for amylase turnover as in (a). The dotted line (\circ) is obtained by extrapolating the steady-state fall-off back to zero time and subtracting the resulting line from the observed data.

time. This line is represented as Q_r in Figure 6 a. The linearity of this total body curve indicates that the labelled enzyme is being removed at a constant rate at least over 6 hours. Ideally the experiment should be continued over several days and the amount of tracer excreted in the faeces determined also.

Knowing the quantity of enzyme in the plasma and the total amount in the body, the difference must be extravascular. This curve is represented as Q_e (Fig. 6 a). It can be seen that after 6 hours $\sim 20\%$ of the dose is still in the plasma, and $\sim 20\%$ has been excreted in the urine; this means that $\sim 60\%$ has entered the extravascular region. The plasma disappearance curves for both dogs were similar and again could be resolved into at least two components. One such result, is shown in Figure 6 b. Due to the rapid metabolic clearance of amylase ($t_{1/2} = 262$ min; Table 5) identification of a third component in the plasma disappearance curve would not be possible without extending the experiment over a longer time period.

The theory of tracer experiments using ^{131}I -labelled plasma proteins was presented by Matthews (26). Our results appear to fit the simplest model, i.e. an open two-compartment system. The rate constants (Table 5) are similar to those obtained for the baboon (12). The enzyme had a distribution half-life of 49.5 min and a metabolic half-life of 262 min (mean of two experi-

Tab. 5. Rate constants for plasma ^{125}I -labelled amylase turnover in the dog

k_1	k_2	k_3	$t_{1/2}$ distribution	$t_{1/2}$ metabolic clearance
$[\text{min}^{-1}]$	$[\text{min}^{-1}]$	$[\text{min}^{-1}]$	$[\text{min}]$	$[\text{min}]$
-0.003185	-0.00987	-0.004095	49.5	262

ments). At raised plasma amylase levels the enzyme had a shorter distribution half-life, i.e. 23 min and a metabolic half-life of 234 min (mean of 3 experiments).

Renal Clearance

A summary of the data on amylase and creatinine excretion at normal serum amylase levels is shown in Table 6. The ratio $C_{\text{am}}/C_{\text{cr}}$ represents the rate at which amylase was cleared relative to creatinine. When amylase excretion was measured as enzyme activity very low values were obtained i.e. in the range 0.002–0.005 % for Dog 1 and 0.11–0.15 % in the case of Dog 2. When amylase clearance was measured as radioactivity the $C_{\text{am}}/C_{\text{cr}}$ ratio reached a plateau of 5.3 % after 4 hours in the case of Dog 1 and 4 % in the case of Dog 2. A plot of the clearance of ^{125}I counts and enzyme activity against time, both corrected for simultaneously measured creatinine clearance, at normal serum amylase levels (a) and at raised serum levels (b) in the same dog, is shown in Figure 7. Enzyme activity was minimal while ^{125}I counts were excreted at a rate 3.5–5 % of the creatinine clearance. The ^{125}I counts excreted, in both cases, were shown to be over 95 % dialysable,

Tab. 6. Data on amylase and creatinine excretion in two tracer experiments

Time period	Serum amylase [U/l]	Urine amylase [U/l]	$C_{\text{am}}/C_{\text{cr}}$ [%]	$C_{\text{am}}^{125}\text{I}/C_{\text{cr}}$ [%]
DOG 1				
0–1 h	1475	15.3	0.005	1.33
1–2 h	1410	4.9	0.002	2.22
2–3 h	1385	4.0	0.002	3.5
3–4 h	1360	1.15	0.003	5.25
4–5 h	1385	2.4	0.005	5.3
5–6 h	1410	5.25	0.004	5.1
DOG 2				
0–1 h	1930	700	0.132	0.87
1–2 h	1900	520	0.107	1.83
2–3 h	1880	520	0.16	3.4
3–4 h	1860	740	0.153	3.45
4–5 h	1820	560	0.118	3.9
5–6 h	1810	270	0.095	4.6

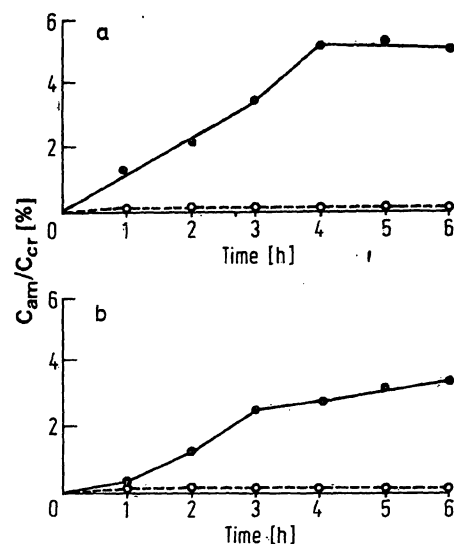


Fig. 7. Renal clearance of amylase activity (C_{am}) (○) and ^{125}I -labelled amylase, measured as radioactivity (●) in the dog, both corrected for simultaneously measured creatinine clearance (C_{cr}) at normal plasma amylase levels (a) and at raised (5–10 fold) plasma amylase levels (b).

and were not precipitable with trichloroacetic acid; the urinary counts, therefore, must represent degraded amylase.

Discussion

The purpose of this investigation was to establish a suitable method for labelling α -amylase which fully preserves the catalytic activity of the enzyme. The chloramine-T method (4) has been used in labelling many enzymes mostly for radioimmunoassay (5–9). As the immunological sites are usually different from the catalytic sites (5, 27, 28) it would be possible to achieve a labelled product suitable for radioimmunoassay, with intact immunological sites, but with great loss of conformity associated with alteration in catalytic activity.

The loss of catalytic activity of α -amylase on treatment with chloramine-T (1 $\mu\text{g}/\mu\text{g}$ enzyme) has been demonstrated here. The method (4) would therefore be unsuitable in labelling α -amylase for in vivo studies due to the associated conformity change. A similar sensitivity to chloramine-T was found in the case of fructose 1,6 diphosphatase (6).

The main advantage of the lactoperoxidase method is that it can give a labelled amylase with 100 % catalytic activity. The loss of enzyme activity obtained at high iodide/amylase molar ratios (≥ 0.8) was obviously caused by some product of the iodination reaction, probably molecular iodine; I_2 has been shown to inhibit α -amylase by combining with internal sulphhydryl groups (2). Preservation of catalytic activity was probably also facilitated by the low concentration of

hydrogen peroxide and radioactivity used. The fact that tyrosine residues are unlikely to be involved in α -amylase catalysis (29) is also fortunate. Since tyrosine must form a complex with lactoperoxidase in the iodination reaction (30) it is likely that tyrosine residues on the outer surface of the molecule only are labelled. Clearly, the large size of the lactoperoxidase would prevent its binding with internal tyrosine residues.

The variables studied in this investigation were amylase concentration and iodide/amylase molar ratios. Ideally, in order to determine optimal conditions for iodination of a given protein, one should investigate the effects of all parameters including concentrations of lactoperoxidase, H_2O_2 , KI, protein, iodide/protein ratio and buffer system. Preliminary experiments established that α -amylase was stable over a concentration range 0.15–1.5 mg/ml, in the presence of 10 μ g/ml lactoperoxidase, 90 μ mol/l H_2O_2 , 10 μ mol/l KI, 5–10 μ mol/l KI, 5–10 μ Ci¹²⁵I in phosphate buffer pH 7.0. These were the components for the iodination of IgG (19). While the concentration of H_2O_2 used was optimal for lactoperoxidase at pH 7.0 (30) it might be possible to increase the efficiency of the iodination of α -amylase by using a lower concentration of H_2O_2 and lactoperoxidase. The efficiency of iodination of IgG was increased by using 18 μ mol/l H_2O_2 and 2 μ g/ml lactoperoxidase (20).

The lowest concentration of amylase at which iodination was attempted was 0.15 mg/ml. While some enzymes are unstable in very dilute solutions without the addition of carrier protein (31) the absence of carrier protein in the iodination reaction did not, per se, cause inactivation since the dilution of amylase was not high enough to cause irreversible denaturation. As mentioned above, a control experiment showed that α -amylase, at a concentration of 0.15 mg/ml for at least 30 minutes retained full catalytic activity. However, inactivation by dilution might be a factor in the case of other enzymes.

The use of insolubilised lactoperoxidase has a number of advantages over the soluble enzyme. It can be readily removed from the iodination reaction by centrifugation. It is also possible to re-use the solid state enzyme (20). Iodination can be performed at 4°C with high efficiency, a factor which may be advantageous in the case of enzymes that are unstable at room temperature.

The results presented here show that α -amylase labelled by Sepharose bound lactoperoxidase, behaved in a similar manner to unlabelled enzyme in both in vitro and in vivo studies. With the necessary care, the method should prove useful for other enzymes which contain accessible tyrosine residues, provided the latter are not involved in catalysis.

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