ACTIVATION OF CHICKEN PEPSIN BY MODIFICATION OF ITS CYSTEINE RESIDUE WITH *o*-NITROPHENYL SULFENYL CHLORIDE

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Received 20 July 1973

1. Introduction

The proteolytic activity of chicken pepsin is similar to that of porcine pepsin [1] but small synthetic peptides, readily hydrolyzed by porcine pepsin, are either not detectably hydrolyzed by the chicken enzyme [2] or are hydrolyzed at a rate which is 10^2–10^3 smaller than by the pig enzyme [3]. Chicken pepsin is a single polypeptide chain of molecular weight 35 000. It is the only pepsin known to contain a free sulfhydryl group [1, 4].

In the present report we show that sulfenylation of the free sulfhydryl group in chicken pepsin with *o*-nitrobenzene sulfenyl chloride (NPS-Cl) [5] results in marked enhancement of the activity of the enzyme towards small synthetic peptides without affecting its proteolytic activity.

2. Materials and methods

2.1. *Materials*

Porcine pepsin (twice crystallized, Lot PM712) and hemoglobin (substrate Hb) were purchased from Worthington. NPS-Cl (Eastman Organic Chemicals), DTNB (Sigma) and the peptide Z–His–Phe–Phe–OEth (Merck) were obtained commercially. Silica gel plates (DC-Karten SIF) were purchased from Riedel-De Haen (Hannover). Chicken pepsin was prepared from chromatographically pure chicken pepsinogen [1]. The peptide Z–Ala–Ala–Phe–Phe–OP4P was synthesized according to the procedure of Sachdev and Fruton [6]. The peptide gave a single spot on TIC (R_f = 0.7, butanol:acetic acid:water 4:1:4, upper phase). After hydrolysis by porcine or chicken pepsin it gave only two products identified as Z–Ala–Ala–Phe and Phe–OP4P (R_f = 0.85 and R_f = 0.25 respectively, in the above solvent).

2.2. *Sulfenylation of pepsins*

Powdered *o*-nitrobenzene sulfenyl chloride was added to a stirred solution of the enzyme (0.15 μmoles/ml) in 0.05 M KCl/HCl buffer pH 2.0, at room temperature (24 ± 2°C). The reaction was followed by measuring optical absorbance at 358 nm of aliquots after centrifugation. As NPS-Cl is practically insoluble under these conditions the absorbance at 358 nm is due to protein-bound NPS. The amount of bound NPS was computed from ϵ_{358} = 4000 M^{-1} cm^{-1} [7]. The modified protein was purified by gel filtration on Sephadex G-25 (1.5 × 20 cm) with 0.5 M HCl/KCl buffer pH 2.0 as eluent.

To remove the NPS group from cysteinyl residues [7] a solution of the modified protein (2 mg/ml) in 0.1 N NaOH was stirred for 30 min at room temperature under nitrogen. The protein was then isolated by gel filtration on Sephadex G-25 (2 × 30 cm) with 0.1 M Tris buffer pH 8.2 as eluent.

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Abbreviations:

DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); NPS-Cl, 2-nitrobenzene sulfenyl chloride; OP4P, 3-(4-pyridyl)-propyl-1-oxy; DMF, dimethylformamide.

2.3. *Pepsin assays*

2.3.1. Peptide activity

Aliquots of stock solutions of the peptides in DMF were added to dilute solutions of HCl and the pH was adjusted to the desired value with 6 N HCl. Substrate concentrations were 5×10^{-5}–1×10^{-3} M, and the final DMF concentration was < 5%. Hydrolysis was initiated by the addition of 5–10 μl of an enzyme stock solution (0.08–8 mg/ml in 0.03 M HCl) and was monitored by an automatic ninhydrin method with a Technicon autoanalyzer [8].

The peptide Z–His–Phe–Phe–ORth (1.3 μmoles/ml) was incubated at pH 2, 3, 4, 5 and 6 (pH 2 buffer KCl/HCl, pH 3, 4, 5 and 6 acetate buffers with native and modified enzyme (20 μg/ml) at 25°C. Aliquots were analyzed after 1, 2 and 4 hr by the ninhydrin metjod. The modified enzyme gave a maximum value of products at pH 4, whereas negligible (< 1% per 4 hr) cleavage was obtained with native enzyme under all conditions.

2.3.2. Proteolytic activity

Assays with denatured hemoglobin as substrate were carried out at pH 1.8 [1]. To determine the apparent K_m and V_m the concentration of hemoglobin was varied from 0.7 to 20 mg/ml.

2.3.3. Milk clotting

Clotting power was determined on reconstituted milk at pH 6.3 [9].

2.4. *Analytical procedures*

Enzyme concentrations were computed from optical absorbance at 280 nm using $A_{280}^{0.1\%} = 1.46$ for both chicken [1] and porcine pepsin [10]. As the absorbance at 280 nm is changed on modification with NPS-Cl the concentrations of modified enzymes were computed from amino acid analyses, of aliquots and published values, for the composition of the proteins [11].

Amino acid analyses were performed on acid hydrolyzates with a Beckman 121 analyzer. Free sulfhydryl groups were determined spectrophotometrically by reaction with DTNB [12].

3. Results

When NPS-Cl was added to a solution of chicken pepsin at pH 2, the solution turned yellow, and spectrophotometric examination revealed the appearance of an absorption band with a peak at 358 nm, indicating sulfenylation of the protein [5]. The absorption at 358 nm leveled off after about 20 min of reaction and remained unchanged on stirring the reaction mixture for an additional 3 hr and several additions of NPS-Cl. The final absorption was proportional to protein concentration and corresponded to an extinction of 4×10^3 cm^{-1} per mole of protein, showing that one NPS residue was incorporated into the protein molecule.

Sulfenyl chloride has been shown to react with tryptophanyl and cysteinyl residues of proteins in acid media [5]. To test whether a tryptophanyl or a cysteinyl residue was sulfenylated in chicken pepsin, a sample of the modified protein was kept for 30 min in solution in 0.1 N NaOH, the dialyzed against water. It was found that treatment with alkali removed quantitatively the NPS group which absorbs around 360 nm, indicating that only a cysteinyl group and no tryptophan residues were sulfenylated [7]. This was substantiated by following the disappearance of the free SH group of the protein on reaction with NPS-Cl. The amount of NPS bound was determined spectrophotometrically at 358 nm, and the amount of free SH was determined by reaction with DTNB. The results (fig. 1) show 1:1 stoichiometry between incorporated reagent and reacted SH group.

The effect of modification of the enzymic properties of chicken pepsin was determined using $Z–Ala_2–Phe_2–OP4P$ [6], Z–His–Phe–OEth, denatured hemoglobin, and recobstituted milk as substrates. Modification, causing a 9-fold increase in the catalytic constant k_{cat}/K_m of chicken pepsin towards the synthetic peptide, had no effect on the activity towards hemoglobin and on milk clotting power of the enzyme (table 1). The pH-dependence of the catalytic activity of the modified enzyme towards the synthetic peptide was found to be identical to that of the native enzyme (fig. 2).

When the NPS modified pepsin was treated with dithioerythritol (150 moles per mole protein, in 0.1 M phosphate buffer pH 7.0, for 15 min), the activity towards the synthetic peptide was found to revert to that of the native enzyme. It thus appeared that the asymmetric disulfide bond linking the NPS to the protein can be reduced by thiols, and the blocking group removed.

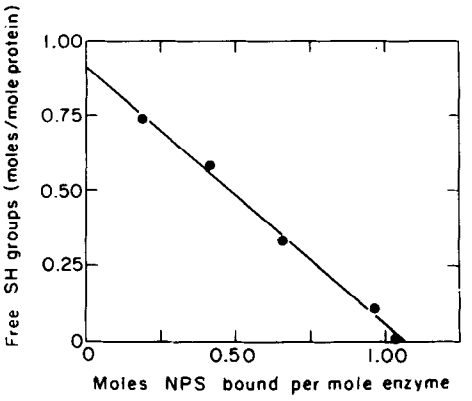

Fig. 1. Correlation between NPS groups incorporated into chicken pepsin and disappearance of free SH groups. NPS groups incorporation was determined spectroscopically at 358 nm and free SH groups were determined with DTNB.

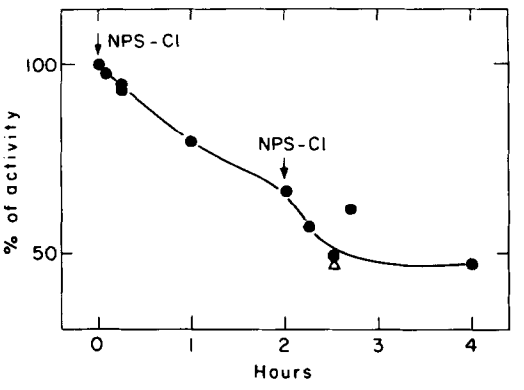

Fig. 3. Decrease in the activity of porcine pepsin on reaction with NPS-Cl at pH 2 and 25°C, (•) activity assayed against Z–Ala_2–Phe_2–OP4P, (△) activity against hemoglobin.

For comparison we studied the reaction of porcine pepsin with NPS-Cl. Porcine pepsin was partially inactivated by NPS-Cl (fig. 3). Incorporation of 1 mole of NPS per mole porcine pepsin decreased the K_{cat}/K_m values (Z–Ala_2–Phe_2–OP4P as substrate, pH 1.5, 25°C) from 4.1×10^6 M^{-1} sec^{-1} to 2×10^6 M^{-1} sec^{-1}. Exposure of NPS-porcine pepsin to 0.1 M alkali did not remove the NPS, indicating that tryptophan was modified in this enzyme.

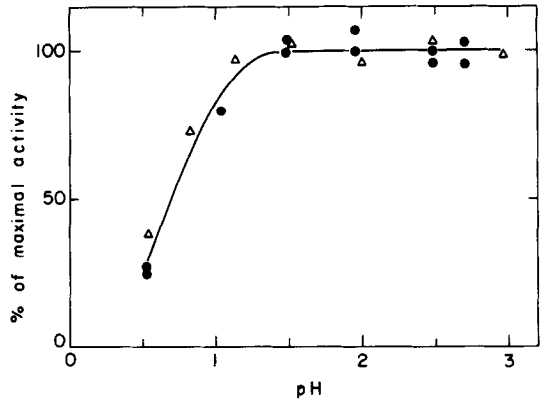

Fig. 2. pH Dependence of k_{cat}/K_m values for the hydrolysis of Z–Ala_2–Phe_2–OP4P by chicken pepsin (•) and NPS modified chicken pepsin (△).

4. Discussion

The equivalence between NPS incorporation and sulfhydryl group disappearance, the susceptibility of the product to alkali, and the reversion of the reaction by dithioerythritol led us to conclude that at pH 2 NPS-Cl selectively sulfenylated the single sulfhydryl group of chicken pepsin. This modification resulted in enhanced catalytic activity towards small synthetic substrates. The values of k_{cat}/K_m for the substrate Z–Ala–Ala–Phe–Phe–OP4P increased 9-fold and the value for the substrate Z–His–Phe–Phe–OEt which was too small to be measured for the native enzyme increased to 40 M^{-1} sec^{-1} on modification. These changes are apparently not a consequence of the modification of groups in the active site of the enzyme as it does not affect the pH dependence of the reaction nor does it affect the proteolytic and milk clotting activities of the enzyme. The enhancement of reactivity towards small peptides may be explained by assuming that the 2-thionitrophenyl group introduced into the enzyme molecule causes the enzyme to change into a conformational state energetically nearer to the one it assumes during the catalytic reaction [13]. During reaction with larger substrates this conformation is perhaps achieved through the binding of groups in these substrates, which are relatively remote from the site of cleavage [14].

Recent studies have shown that covalent modifica-

Table 1
Enzymic activity of chicken pepsin modified with NPS-Cl.

Substrate	Kinetic constants	Native	Modified
S–Ala_2–Phe_2–OP4P[a]	$k_{cat}(sec^{-1})$	20.3	29.6
	$K_m(M^{-1})$	1.9×10^{-2}	0.32×10^{-3}
	$k_{cat}/K_m(M^{-1}\ sec^{-1})$	1.1×10^4	9.2×10^4
Z–His–Phe–Phe–OEth[b]	$k_{cat}/K_m(M^{-1}\ sec^{-1})$	<0.1	40
Hemoglobin[c]	$V_m(\Delta A_{280}/min)$	0.27	0.26
	K_m(mgHb/ml)		5.0
Milk clotting[d]	%	100	86

[a] Hydrolysis of Z–Ala_2–Phe_2–OP4P was measured at pH 1.5 and 25°C with an enzyje concentration of 9.1×10^{-9} M.
[b] Hydrolysis of Z–His–Phe–Phe–OEth was measured at pH 4.0 and 25°C with an enzyme concentration of 20 μg/ml.
[c] Hydrolysis of hemoglobin at different concentrations was measured at pH 1.8 and 37°C, by measurements of the TCA soluble-products with an enzyme concentration of 0.99×10^{-7} M. The results were computed from a Linewaver–Burke plot using substrate concentrations expressed in mg/ml and velocities expressed in change of A_{280}/min.
[d] Milk clotting was measured on reconstituted milk at 30°C, and the results computed by the method of Berridge [9].

tion of enzymes is an important mode of regulating their activity in living systems [15]. Enzyme-catalyzed phosphorylation of seryl residues for example has been shown to change the catalytic of phosphorylase, glycogen synthetase and pyruvate dehydrogenase [15]. The change in the catalytic parameters of chicken pepsin by the chemical modification of a single residue is one of the first synthetic reactions of this kind.

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