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PYRROLOOXYGENASE: ITS ACTION ON TRYPTOPHAN-CONTAINING ENZYMES AND PEPTIDES*

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SUMMARY

1. A new type of enzyme named pyrrolooxygenase, which oxidizes the pyrrole ring of tryptophanyl residues in proteins, is described. It inhibited the activity of chymotrypsin (EC 3.4.4.5), trypsin (EC 3.4.4.4), pepsin, (EC 3.4.4.1), lysozyme (EC 3.2.1.17) and soybean trypsin inhibitor.

2. Spectrophotometric determinations indicated that the tryptophanyl residues of the hydrolytic enzymes were attacked by the pyrrolooxygenase.

3. Studies with two synthetic peptides, a pentapeptide and a nonapeptide containing a single residue of tryptophan, indicated that they were substrates of the pyrrolooxygenase, which transformed the tryptophan into kynurenine derivatives.

4. When ethyl indole-3-propionylglycinate was used as a substrate, it was found that the indole ring was oxidized to 2-aminophenacyl derivatives but the peptide bond was not cleaved. This pyrrolooxygenase was identical in its properties with the previously described tryptophan pyrrolooxygenase.

INTRODUCTION

In a previous paper[1] we described the existence of a new type of enzyme which was named pyrrolooxygenase which oxidized simple substituted tryptophans such as ethyl *N*-acetyltryptophan. The products formed were ethyl 2-acetamido-3-(2'-formamidobenzoyl)-propionate and its deformylated derivative (ethyl *N*-acetylkynurenine) which was formed by a formylase present in the enzymatic preparation. Pyrrolooxygenase activity was then examined on tryptophan containing peptides and enzymes, where the oxidation of the tryptophyl residue could lead to an inhibition of the enzymatic activity. It is well known that in many hydrolytic enzymes containing tryptophan, the oxidation of the latter by chemical agents (*e.g. N*-bromosuccinimide) led to an inactivation of the former[2]. The action of pyrrolooxygenase on the same enzymes had the same inhibitory effect as *N*-bromosuccinimide oxydation[3]. When the hydrolytic enzymes were isolated after pyrrolooxygenase attack, a marked decrease in their tryptophan content was found.

There are very few examples of enzymatic interactions between oxygenases

* Dedicated to Professor V. Deulofeu on his seventieth birthday.

and protein. The best known is the oxidation of the proline residues of protocollagen by proline hydroxylase, which results in the formation of collagen[4]. Apparently the action of the hydroxylase is independent of the chain length or conformation of the protein, since it also oxidized small synthetic peptides containing proline[5]. This was also the case with pyrrolooxygenase. It oxidized the tryptophan residues of the hydrolytic enzymes and also of tryptophan containing synthetic peptides. We had already mentioned[1], that the enzyme did not attack other proteic aminoacids, and this was confirmed by hydrolysis of the pyrrolooxygenase oxidized peptides, since all the original aminoacids were recovered unchanged, except for kynurenine formed at expense of the tryptophan.

MATERIALS AND METHODS

Trypsin (EC 3.4.4.4), chymotrypsin (EC 3.4.4.5), lysozyme (EC 3.2.1.17), pepsin (EC 3.4.4.1), papain (EC 3.4.4.10), soybean trypsin inhibitor, *N*-bromosuccinimide, sodium dithionite, L-tryptophan and bovine serum albumin were commercial samples of analytical grade. Ethyl indole-3-propionylglycinate (I) and indole-3-propionylglycine were prepared by synthesis[6]. The pentapeptide, 5-carboxy-2-pyrrolidone-Trp–Pro–Arg–Pro (II) and the nonapeptide, 5-carboxy-2-pyrrolidone-Trp–Pro–Arg–Pro–Gln–Leu–Pro–Pro (III) were a generous gift from Dr M. A. Ondetti (New Brunswick, N.J.). All other reagents were of analytical grade. Wheat germ was a gift from Molinos Rio de la Plata. Male Wistar Albino rats weighing between 200 and 250 g were used. Solvents used for chromatography were butanol–acetic acid–water (4:1:5, by vol.) (Solvent I); chloroform–methanol–17% ammonia (2:2:1, by vol.) (Solvent II) and phenol–water (7:3, by vol.) (Solvent III).

When a product analysis of the oxidized pentapeptide and nonapeptide was made, two of the indicated incubation mixtures containing 20 μg of substrate (see *Assay of pyrrolooxygenase*) were pooled, evaporated to dryness and the products were extracted with methanol. Blanks were run simultaneously omitting either enzyme or sodium dithionite and treated in the same manner. The methanol was evaporated and the residues were separated by thin-layer chromatography on cellulose using Solvent I. The products were located either by spraying with Ehrlich's reagent (yellow-orange colour) or by their fluorescence under ultraviolet light. The bands corresponding to the products or to the original peptides were eluted with 30% acetic acid and the eluate evaporated to dryness. The residues were hydrolyzed by heating with 6 M hydrochloric acid at 110 °C for 20 h in a vacuum sealed tube. The hydrolysis products were separated by paper chromatography on Whatman No. 1 (Solvent I) or by two-dimensional chromatography on thin-layer chromatography (silica Gel G) using Solvent II first followed by Solvent III, as developers.

Wheat germ and rat liver pyrrolooxygenases were extracted and purified as described[1]. Rat liver microsomes were prepared as indicated[1]. Unless otherwise stated, the DEAE purified fractions were used.

Assay of pyrrolooxygenase

Unless otherwise indicated, pyrrolooxygenase activity toward tryptophan containing enzymes or peptides was assayed as follows: 10 to 20 μg of the crystalline enzyme or peptide, 10 μmoles of phosphate buffer (pH 7.4), 0.05 μmole of sodium

dithionite and pyrrolooxygenase (5 to 20 μg of protein) were incubated in a final volume of 100 μl for 15 to 30 min at 37 °C. Two blanks were run concomitantly omitting either dithionite or pyrrolooxygenase. The oxidized hydrolytic enzymes: trypsin, chymotrypsin, pepsin and papain were then assayed for proteolytic activity on bovine serum albumin by the method of Folin–Ciocalteu[7]. When lysozyme was oxidized, its lytic activity was assayed on *Micrococcus lysodeikticus* by turbidimetry[8]. Soybean trypsin inhibitor was assayed by its inhibitory activity on trypsin. Potato phosphorylase was assayed as described[9]. Pyrrolooxygenase activity on indole-3-propionylglycine was assayed by incubating 43 μmoles of the acid (or its ethyl ester) under the above described conditions and measuring substrate consumption with Ehrlich's reagent (2% *p*-dimethylaminobenzaldehyde in glacial acetic acid–perchloric acid (84:16, by vol.) at 552 nm. Protein was estimated at 280 nm. A Beckman DU spectrophotometer was used for all the spectrophotometric determinations. Fluorimetric analyses were performed with an Aminco Bowman spectrofluorimeter.

TABLE I

EFFECT OF PYRROLOOXYGENASE ON THE ACTIVITY OF TRYPTOPHAN CONTAINING PROTEINS

Incubation mixtures and activity determinations were as indicated in Materials and Methods. The pyrrolooxygenase used, unless otherwise indicated, was the DEAE purified wheat germ fraction.

Compound	*Preincubated with*	*Remanent activity (%)*
Lysozyme	—	100
	N-Bromosuccinimide	None
	Pyrrolooxygenase + dithionite	None
Pepsin	—	100
	N-Bromosuccinimide	25
	Pyrrolooxygenase + dithionite	30
Chymotrypsin	—	100
	N-Bromosuccinimide	27
	Pyrrolooxygenase + dithionite	31
	Pyrrolooxygenase[a] + dithionite	40
Papain	—	100
	N-Bromosuccinimide	100
	Pyrrolooxygenase + dithionite	100
Trypsin	—	100
	N-Bromosuccinimide	5
	Soybean trypsin inhibitor	None
	Pyrrolooxygenase + dithionite	5
Soybean trypsin inhibitor	—	100[b]
	Pyrrolooxygenase[c] + dithionite	None
	N-Bromosuccinimide[d]	None
Potato phosphorylase	—	100
	N-Bromosuccinimide	100
	Pyrrolooxygenase + dithionite	100

[a] Rat liver enzyme (30–70% ammonium sulphate fraction[1]) was used.

[b] 100% of soybean trypsin inhibitor activity represents a total inhibition of tryptic activity under the conditions described in Materials and Methods.

[c] Pyrrolooxygenase and dithionite were not removed before adding trypsin.

[d] *N*-Bromosuccinimide was removed before adding trypsin to assay activity of soybean trypsin inhibitor.

RESULTS

Inhibition of enzymes

The activities of lysozyme, trypsin, chymotrypsin, pepsin and soybean trypsin inhibitor, were strongly inhibited by incubation with pyrrolooxygenase. This inhibition paralleled the inhibition obtained by incubating with *N*-bromosuccinimide under controlled conditions[2] (Table I). Enzymes which did not contain tryptophanyl residues essential for their catalytic activity, *e.g.* potato phosphorylase and papain, were not inhibited by pyrrolooxygenase. All the inhibited enzymes contained one or more essential tryptophanyl residues, which when oxydized with *N*-bromosuccinimide under the described conditions[2] afforded an inactive enzyme. Since pyrrolooxygenase oxidized ethyl *N*-acetyl tryptophan, its inhibitory effect on the hydrolytic enzymes could be attributed to the same reaction. This was supported by the fact that the absorbance at 280 nm and the fluorescence at 350 nm decreased in the oxidized enzymes. When a pyrrolooxygenase oxidized trypsin was isolated from the incubation mixture by filtration through Sephadex G-75 and its loss in hydrolytic activity was measured, it was found that it went together with a decrease in its absorbance at 280 nm and a decrease in its fluorescence at 350 nm (Fig. 1).

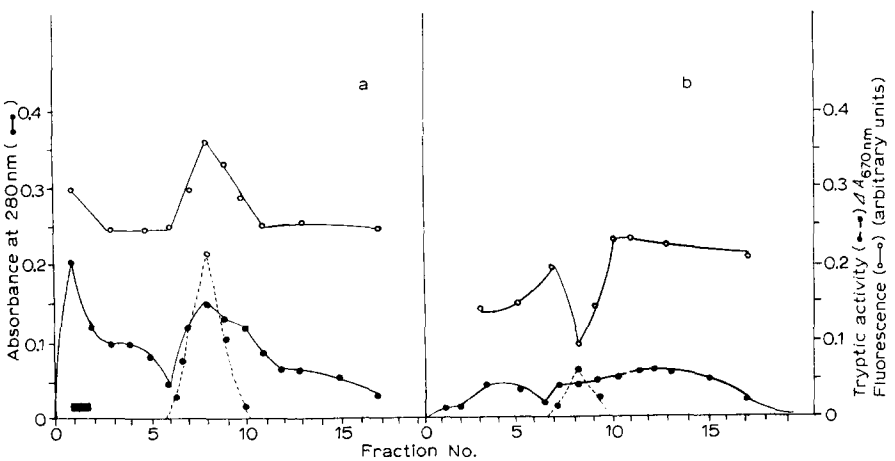

Fig. 1. Effect of pyrrolooxygenase on trypsin, as measured by tryptic activity and the decrease in fluorescence and absorbance. (a) The reaction mixture containing 0.1 mg of trypsin, pyrrolooxygenase (50 μg of protein), and buffer, in a final volume of 300 μl, was incubated for 30 min at 37 °C and then filtered through a Sephadex G-75 column (1 cm × 25 cm). The activities of trypsin and pyrrolooxygenase were assayed as described. Fluorescence of tryptophan at 350 nm was measured with an Aminco Bowman spectrofluorimeter. ▬, pyrrolooxygenase activity. (b) The incubation mixture and the conditions were the same as above, except for the addition of sodium dithionite (0.5 mM) to the incubation mixture.

As can be seen in Table I, the oxidation of soybean trypsin inhibitor by pyrrolooxygenase prevented the oxidation of the trypsin which was added later. This was due to the inactivation of pyrrolooxygenase during the first oxidation process. Pyrrolooxygenase was not inactivated when preincubated alone without addition of substrate in the presence of sodium dithionite, hence it did not act on itself. The

absorbance at 280 nm of the pyrrolooxygenase preparations decreased after its preincubation with sodium dithionite, although the enzymatic activity was not affected. These results indicated that pyrrolooxygenase itself did not contain essential tryptophans.

The effects of enzyme concentration and pH on chymotrypsin inactivation are shown in Fig. 2 and Fig. 3.

Pyrrolooxygenase activity toward hydrolytic enzymes was also found in rat liver extracts (Table I). The natural reducing agent for the mammalian pyrrolooxygenase was found to be NADPH and an electron transport system which required microsomes (where the pyrrolooxygenase was localized[1]) and the 105 000 × *g* supernatant (Table II).

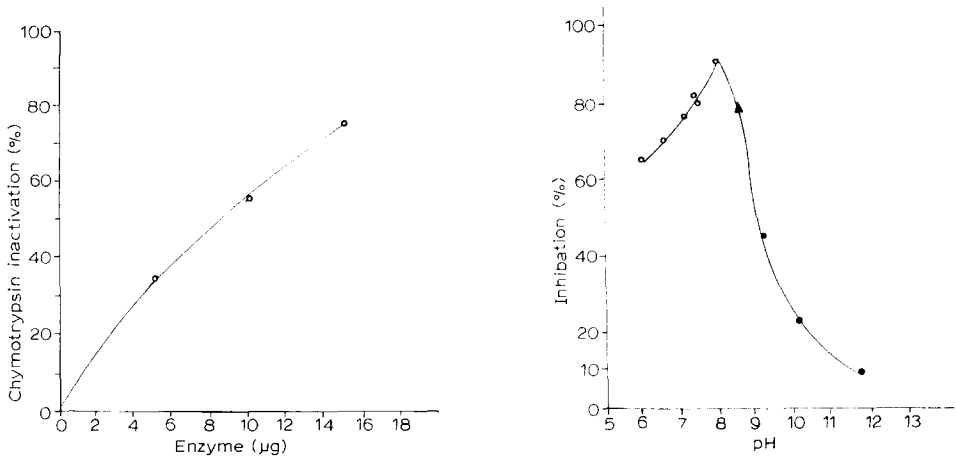

Fig. 2. Effect of pyrrolooxygenase concentration on chymotrypsin inactivation. Chymotrypsin (20 μg) was preincubated with the indicated amount of purified wheat germ pyrrolooxygenase *plus* dithionite. Chymotrypsin activity was assayed as described. Preincubated time was 15 min

Fig. 3. Influence of pH on pyrrolooxygenase activity. The incubation mixture contained in a final volume of 100 μl: 10 μmoles of the indicated buffer at the indicated pH, 20 μg of chymotrypsin, wheat germ pyrrolooxygenase and dithionite. The incubations were run for 30 min at 37 °C. Chymotrypsin incubated at the same pH without pyrrolooxygenase was taken as 100% of activity. Chymotryptic activity was assayed as described. ○—○, phosphate buffer; ●—●, glycine buffer; ▲—▲, Tris–HCl buffer.

The oxidized trypsin (Fig. 1) was eluted from the Sephadex column with the same volume as the untreated trypsin. Similar results were obtained with chymotrypsin[3]. This indicated that the hydrolytic enzymes had not been cleaved during the oxidation process, as was the case when *N*-bromosuccinimide oxidations were used[10]. To confirm that no peptide bonds were cleaved during the process, the enzymatic oxydation of ethyl 3-indolepropionylglycinate (I) was examined.

Enzymatic oxidation of ethyl indole-3-propionylglycinate (I). Products formed

Ethyl 3-indolepropionylglycinate (5 × 20 μg) was incubated at 37 °C and pH 7.4 for 120 min with pyrrolooxygenase using the technique described in *Assay of pyrrolooxygenase*. The product formed was extracted with methylene chloride, the

TABLE II

IDENTITY OF THE NATURAL REDUCING AGENT OF RAT LIVER PYRROLOOXYGENASE

Chymotrypsin (20 μg) was preincubated with buffer or with the indicated additions and reducing agents for 30 min at 37 °C, and then assayed for its chymotryptic activity as indicated in *Assay of pyrrolooxygenase.*

Chymotrypsin preincubated with		
Addition	*Reducing agent*	*Remanent chymotryptic activity (%)*
None	—	100
Microsomes + 105 000 × *g* supernatant	—	80
	Sodium dithionite	70
	NADPH[a]	10

[a] The NADPH system contained: NADPH (0.04 μmole), glucose 6-phosphate (0.5 μmole), glucose-6-phosphate dehydrogenase (5 μl), nicotinamide (5 μmoles) and $MgCl_2$ (1 μmole).

organic solvent was washed with water, dried (Na_2SO_4) and then evaporated to dryness. The residue (0.5 mg) was homogeneous by thin-layer chromatography on cellulose (R_F 0.90, Solvent I) when revealed by its fluorescence and by spraying with Ehrlich's reagent (yellow). It was dissolved in 0.1 ml of a molar sodium hydroxide solution in 50% ethanol and kept for 15 h at 25 °C. The solution was evaporated to dryness and the products formed were identified by thin-layer chromatography on cellulose (Solvent I). They were identical with glycine and 3-(2'-aminobenzoyl)-propionic acid (V) (Fig. 4). The latter was obtained by the chemical oxidation of methyl indole-3-propionate (VI) with *p*-nitroperbenzoic acid[1], followed by saponification of the formed methyl 3-(2'-formamidobenzoyl) propionate (VII) (Fig. 5). These results, together with the known properties of pyrrolooxygenase[1], indicated that the compound obtained by the enzymatic oxidation of (I) was ethyl 3-(2'-formamidobenzoyl)propionyl glycinate (IV) (Fig. 4). A further confirmation that the peptide bond is not cleaved during the enzymatic oxidation was also obtained when 3-indolepropionylglycine was enzymatically oxidized with pyrrolooxygenase without liberation of glycine, whereas the oxidation carried out with *N*-bromosuccinimide liberated glycine[6].

Enzymatic oxidation of tryptophan containing peptides

The pentapeptide (II) (20 μg) was oxidized with pyrrolooxygenase as described in Materials and Methods. Two incubation mixtures were pooled, diluted to a final volume of 0.4 ml and the decrease in the absorbance at 280 nm was measured (Fig. 6a). A strong decrease in the absorbance indicated that the tryptophanyl residue was oxidized, and this was confirmed by the decrease in the typical tryptophan fluorescence at 350 nm (Fig. 6b).

Similar results were obtained when the nonapeptide (III) was oxidized under the same conditions (Figs 7a and 7b). The enzymatic oxidation of the tryptophanyl residue of the peptides was substrate dependent (Fig. 8).

To establish the identity of the product formed at expense of the tryptophanyl residue during the enzymatic oxidation, the incubation mixtures containing the oxi-

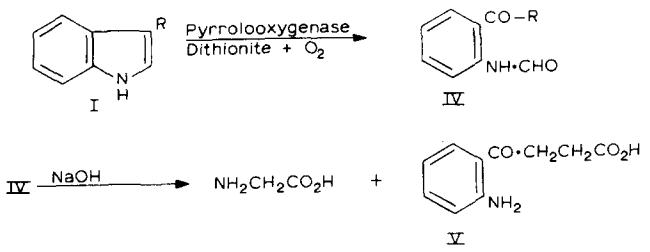

Fig. 4

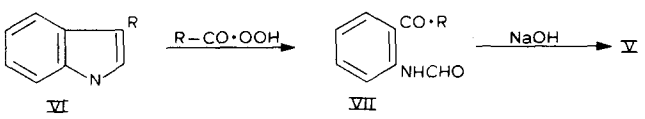

Fig. 5

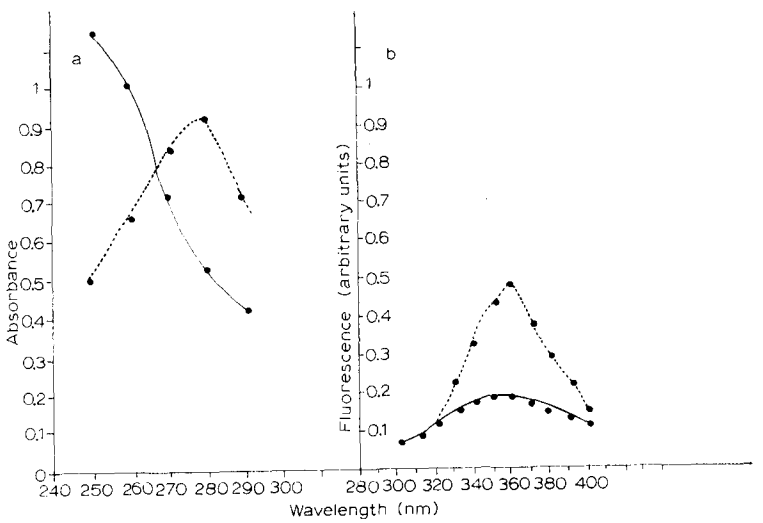

Fig. 6. Oxidation of pentapeptide (II) by pyrrolooxygenase. (a) Ultraviolet spectra of pentapeptide (II) with pyrrolooxygenase (●---●) and with pyrrolooxygenase plus dithionite (●—●). (b) Fluorescence emission spectra of the untreated pentapeptide (pentapeptide *plus* pyrrolooxygenase) (●---●) and the oxidized pentapeptide (pentapeptide *plus* pyrrolooxygenase plus dithionite) (●—●). Excitation was at 290 nm.

dized penta- or nonapeptide were evaporated to dryness and the products extracted and separated by thin-layer chromatography on cellulose as described in Materials and Methods. The pentapeptide (II) (R_{Trp}, 1.4), was transformed into two kynurenine containing peptides corresponding to the formylkynurenine (R_{Trp}, 1.03) and kynu-

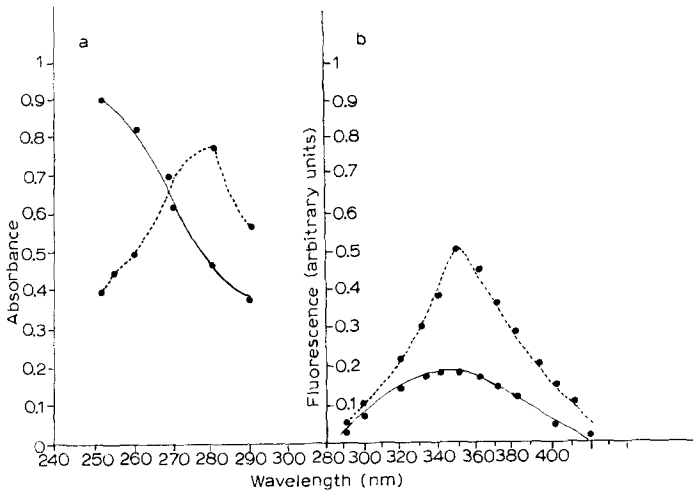

Fig. 7. Oxidation of nonapeptide (III) by pyrrolooxygenase. (a) Ultraviolet spectra of nonapeptide (III) *plus* pyrrolooxygenase (●---●) and pyrrolooxygenase *plus* dithionite (●—●). (b) Fluorescence emission spectra of the untreated nonapeptide (nonapeptide *plus* pyrrolooxygenase) (●---●) and the oxidized nonapeptide (nonapeptide plus pyrrolooxygenase *plus* dithionite) (●—●). Excitation was at 290 nm.

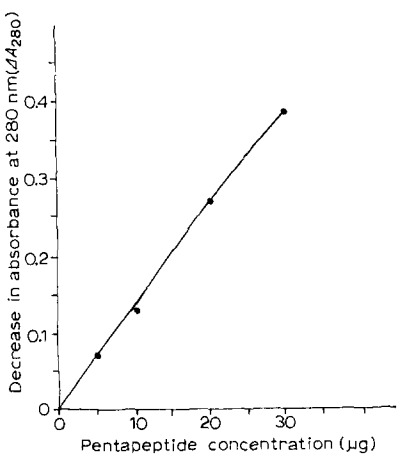

Fig. 8. Effect of substrate concentration on the rate of tryptophan oxidation. The substrate used was pentapeptide (II). The incubation conditions have been previously described. Tryptophan consumption was taken as the difference in the absorbance at 280 nm between a blank, in which the dithionite was omitted, and the complete system.

renine (R_{Trp}, 1.2) type of derivatives, as was the case when ethyl *N*-acetyltryptophan was used as a substrate[1]. When the nonapetide (III) (R_{Trp}, 1.63) was used as a substrate, two oxidized peptides were also isolated with R_{Trp} 1.36 for the formylated peptide and R_{Trp} 1.5 for the deformylated peptide. The oxidized peptides were eluted, hydrolyzed, and the aminoacids separated as described in Materials and Methods. They were identified as proline, glutamic acid, arginine, leucine and (–)-kynurenine, thus indicating that the tryptophan was transformed into kynurenine during the enzymatic oxidation.

DISCUSSION

The enzymatic oxidation of the tryptophan residue of hydrolytic enzymes introduces a new type of chemical modification of a proteic amino acid which may be of biological significance. It is known that the chemical modification of an essential aminoacid in an enzyme can suppress or modify its activity[11]. Thus, the chemical oxidation of tryptophan containing enzymes by different oxidation agents such as *N*-bromosuccininimide, ozone or iodine, led to inactive enzymes due to the specific oxidation of the tryptophanyl residues[2,12]. This type of chemical reagents were termed "target reagents" due to their specificity. Pyrrolooxygenase is the first enzyme to have these properties, introducing a new type of metabolic regulation. The fact that pyrrolooxygenase has the properties of a mixed-function oxidase, requiring both oxygen and a reducing agent, could be the way its activity is regulated *in vivo*. It remains to be established the number and the sequential position of the oxidized tryptophanyl residues in each enzyme.

Pyrrolooxygenase can also be of diagnostic value to establish the presence of an essential tryptophan in enzymes whose sequence has not yet been established. Since pyrrolooxygenase does not oxidize any other proteic aromatic aminoacid and it does not cleave the peptide bond, it could be used as a safer specific reagent for the tryptophanyl residue than many of the chemical reagents employed for that purpose.

The enzyme was isolated from plant an animal sources. In the former it was isolated from wheat germ, but it was also localized in the chloroplasts of spinach and Swiss chard leaves. The mammalian enzyme was localized in rat liver microsomes, (Table II).

It was shown previously[1], that there are more than one pyrrolooxygenases acting on different substrates. We were able to distinguish at least two pyrrolooxygenases, skatole pyrrolooxygenase and tryptophan pyrrolooxygenase. The properties of the pyrrolooxygenase described in this paper are very similar to those of tryptophan pyrrolooxygenase and they are probably the same enzyme.

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REFERENCES

1 R. B. Frydman, M. L. Tomaro and B. Frydman, *Bicohim. Biophys. Acta*, 284 (1972) 63.
2 T. F. Spande and B. Witkop, in C. H. W. Hirs, *Methods in Enzymology*, Vol. XI, Academic Press, New York, 1967, p. 498.
3 R. B. Frydman, M. L. Tomaro and B. Frydman, *FEBS Lett.*, 15 (1971) 305.
4 B. Peterkofsky and S. Udenfriend, *J. Biol. Chem.*, 238 (1963) 3966.
5 J. O'D. McGee, R. E. Rhoads and S. Udenfriend, *Arch. Biochem. Biophys.*, 144 (1971) 343.

6 A. Patchornik, W. B. Lawson, E. Gross and B. Witkop, *J. Am. Chem. Soc.*, 82 (1960) 5923.
7 M. Laskovsky, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. II, Academic Press, New York, 1955, p. 26.
8 D. Shugar, *Biochim. Biophys. Acta*, 8 (1952) 302.
9 E. Slabnik and R. B. Frydman, *Biochim. Biophys. Res. Commun.*, 38 (1970) 709.
10 B. Witkop, *Adv. Protein Chem.*, 16 (1961) 221.
11 H. Holzer and W. Duntze, *Annu. Rev. Biochem.*, 40 (1971) 345.
12 M. A. Raftery and F. W. Dahlquist, in Zechmeister, L. *Forschritt Chemische Organische Naturstoffe*, Vol. XXVII, Springer Verlag, Berlin, 1969, p. 345.