Surface Layer of Wool. I. Dityrosine Synthesis and Characterization

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ABSTRACT: An assay of the enzymic oxidation of tyrosine to dityrosine was performed to determine the optimum conditions for dityrosine formation. Improvements were introduced into the method for dityrosine synthesis and purification so as to obtain a higher yield (10%) of pure dityrosine. The product served as a reliable reference for studies of the dityrosine content of wool. © 1997 John Wiley & Sons, Inc. J Appl Polym Sci **66:** 2359–2363, 1997

INTRODUCTION

This work is the first of a series on the composition of wool and the nature of its surface layer. Initially the study will concentrate on dityrosine.

Dityrosine (2-amino-3-[5′-(2 amino-2-carboxy ethyl)-6,2′-dihydroxybiphenyl-3-yl]propionic acid) is composed of two tyrosine (2-amino-3-[4-hydroxyphenyl]propionic acid) residues which are linked by an *ortho,ortho* biphenyl bond (Fig. 1). It was discovered by Gross and Sizer[1] as a product of the oxidation of L-tyrosine by horseradish peroxidase *in vitro.* They proposed a free radical mechanism in which phenoxy radicals of tyrosine were paired, forming the biphenyl linkage. The trimer (trityrosine) and higher polymers could also be formed by reaction between a phenoxy radical and a dimer which had been oxidized to a free radical. Andersen[2] discovered the natural occurrence of dityrosine when he isolated two fluorescent amino acids, dityrosine and trityrosine, from hydrolysates of resilin. Subsequently, dityrosine was isolated from wool keratin.[3,4]

Dityrosine is a component of insoluble structural proteins. It has been suggested that dityrosine forms covalent protein–protein crosslinks *in vivo,* which are probably made photosynthetically via a peroxidative reaction. The crosslinks are believed to aid the mechanical stability of the biological structure involved. Such a crosslinking reaction has been observed in an *in vitro* system with horseradish per oxidase as shown by Andersen[5] and Aeschbach and colleagues.[6] Andersen[5] found that oxidation of silk fibroin with peroxidase and hydrogen peroxide produced a fluorescent gel that contained dityrosine. The reaction mechanism was similar to the one formulated by Gross and Sizer,[1] but with the oxidation taking place at the level of the protein. Aeschbach and colleagues[6] extended the work and showed that dityrosine formation could be induced by oxidation in nonstructural proteins such as insulin. The oxidation has been shown to proceed with nitration, ozonation, or by radiation-induced dimerization. However it is produced, dityrosine can be detected in acid hydrolysates of protein by its characteristic ultraviolet fluorescence or by amino acid analysis.

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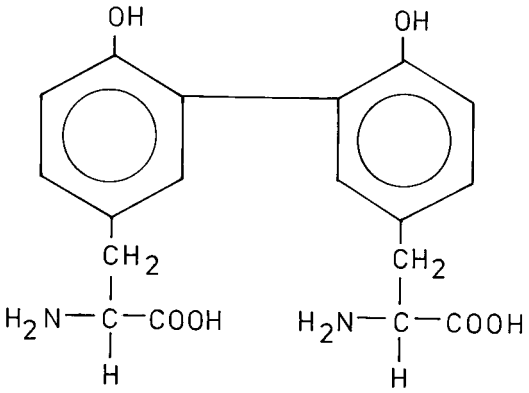

Figure 1 Structure of dityrosine.

Importance of Dityrosine in Wool

The presence of dityrosine in wool keratin is of interest because it is believed to aid in the mechanical stability of protein by functioning as a covalent crosslinkage between peptide chains.[2,7] In order to investigate the properties of dityrosine within wool, it is necessary to first synthesize the dimer so that a reference compound is available. Attempts to synthesize dityrosine chemically have proven unsuccessful.[8] All successful methods employ a two-step process of enzymatic oxidation of tyrosine followed by a purification of the product. Aeschbach and colleagues[6] described a method in which *N,N′*-diacetyldityrosine was synthesized via the peroxide–peroxidase oxidation of *N*-acetyltyrosine. The product was purified by gel filtration chromatography and preparative thin layer chromatography, deacetylated to dityrosine, and further purified. Malanik and Ledvina[9] reported a shorter method with the direct enzymatic-peroxide oxidation of tyrosine followed by purification. Initial work showed the latter to give a better yield and therefore is used here.

Synthesis of Dityrosine

The method of dityrosine synthesis chosen was the horseradish peroxidase catalyzed oxidation of tyrosine[9] as modified by Gargan.[10] However, the yield of dityrosine synthesized by this method was low (2%). The oxidation of phenolic compounds by a peroxidase enzyme is complex and the low yield of dityrosine obtained could be due to a number of causes, such as incomplete oxidation of the tyrosine or the formation of higher oxidation products. In this work, the reaction was studied in order to determine the effect of operating variables on, and to optimize the yield of, dityrosine.

MATERIALS

1. From Sigma (U.K.): horseradish peroxidase (EC 1.11.1.7); type I, 40 units per mg solid; type II, 150 units per mg solid; 41.6 kDa; Folin and Ciocalteau's phenol reagent (F/C).
2. From Pharmacia (U.K.): Sephadex G-10.
3. From BDH (U.K.): L-tyrosine and all other chemicals (AR grade).

PREPARATION OF DITYROSINE

L-Tyrosine (1 g) was dissolved in a solution of 600 mL distilled water, 5 mL of 35% ammonium hydroxide, and 2*M* formic acid, to a pH of 9.2. Hydrogen peroxide (120 μL of 30%) was added and the solution incubated for 1 h at 40°C. Horseradish peroxidase (40 mg) was added and the reaction mixture was incubated with gentle mixing for a further 3 h. The pH was adjusted to 6.0 with 2*M* formic acid, and the solution rotary evaporated at 40°C to about 60 ml. Darco G-activated carbon was stirred in and the solution allowed to stand for 18 h. The solution was filtered, concentrated by rotary evaporation to about 30 mL, and purified using gel filtration chromatography on a Sephadex G-10 column. Column preparation consisted of swelling the gel (40 g/250 mL H_2O) under slow stirring for 3–4 h. Fines were removed and the solution was degassed under vacuum and loaded bubble free into a 40-cm-long, 3-cm-diameter glass tube. The column was allowed to stand under water elutant. To operate the column, the water level was reduced, 5 mL of crude dityrosine was layered on top of the gel, and the bed was eluted from a reservoir at a rate of 6 mL/h. The base outlet of the column was monitored continuously at 254 nm using an LKB 4071A Uvicord spectrophotometer and flow cell. Fractions (5 mL) were collected using a BTL Chomofrac collector.

The spectrometer outlet recorded peaks that were identified using a combination of spiking and one-dimensional descending paper chromatography (3 MM Whatman paper). The solvent system used was *n*-butyl alcohol : acetic acid : water (55 : 15 : 30 vol) under an ammonia vapor atmosphere. The solvent front was allowed to run about 15 cm; the paper was removed, dried, exposed to ammonia vapor, and observed under 254 nm light. Dityrosine showed as a bright blue fluorescence. The paper was then sprayed with either F/C phenol reagent or cadmium–ninhydrin (C/N) reagent and developed. The R_f value (distance traveled

by component/distance traveled by solvent front) was determined because it was characteristic of a given substance under the specific conditions used, and so provided a basis for identification.

A typical elution profile obtained from the Sephadex G-10 column exhibited four peaks corresponding to horseradish peroxidase, trityrosine, dityrosine, and tyrosine. A second purification step was run on the combined lyophilized fractions corresponding to the third peak. The elution profile from this second purification exhibited three peaks corresponding to trityrosine, dityrosine, and tyrosine. The second-peak fractions were pooled, lyophilized, and stored over P_2O_5. The dityrosine synthesized was a hygroscopic white powder that darkened on exposure to air.

Characterization of Dityrosine

The purity of the dityrosine obtained above needed to be ensured before it could be used as a valid reference material. Descending paper chromatography on the dityrosine product gave R_f = 0.32, in agreement with the value for dityrosine given in the literature.[9] Thin layer chromatography on the dityrosine product with silica gel plates and the same solvent system gave R_f = 0.17, which also agreed with the dityrosine value from the literature.[8] The UV absorption spectra obtained with the dityrosine product was found to be typical of an *o,o'*-biphenol compound.[1,11] In addition, the dityrosine product had maxima at 284 nm in acid medium and 316 nm in alkaline medium. Thus the UV adsorption spectra of the dityrosine product showed a typical protonation equilibrium, where the change in UV adsorption maxima occurred at about pH = 7.0. When a solution of the dityrosine product at pH = 9.5 was excited with an excitation wave length of 315 nm, a fluorescence spectrum was obtained with an emission maximum occurring at a wave length of 406 nm, in agreement with literature on dityrosine.[5] In addition, IR spectra and RP-HPLC both confirmed high-purity dityrosine as the product.

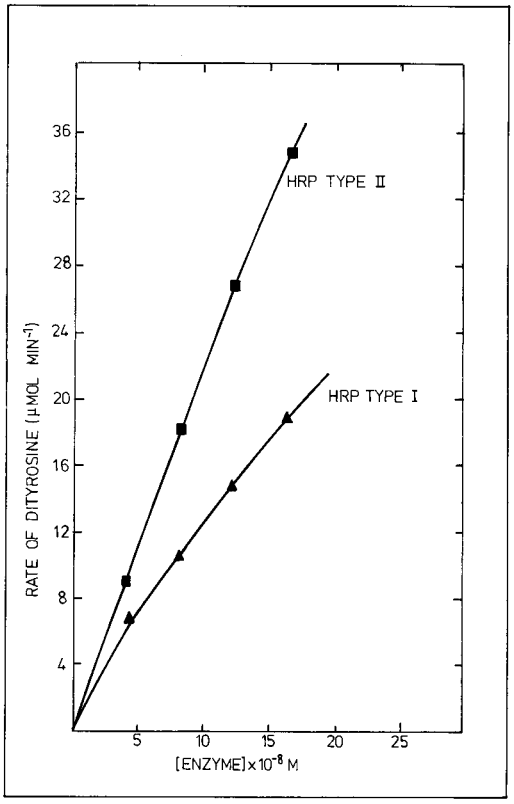

Figure 2 Rate of dityrosine formation as a function of enzyme concentration (types I and II).

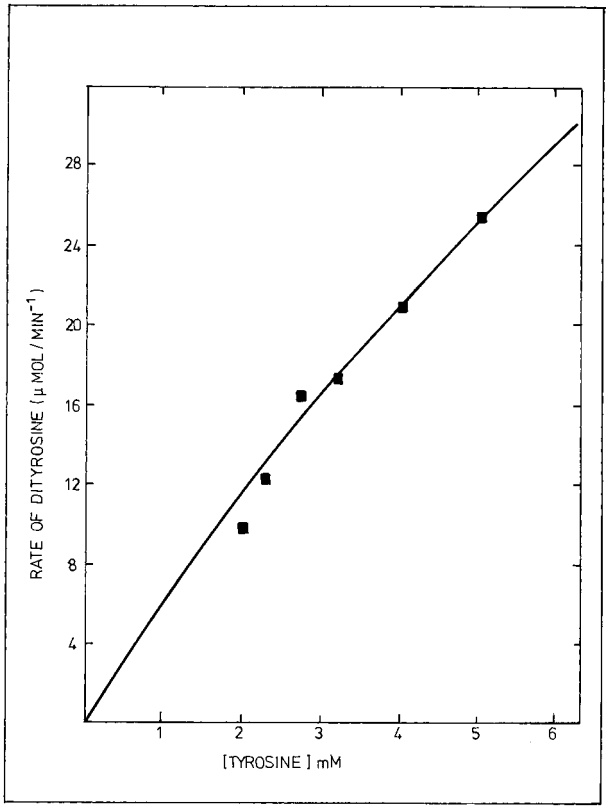

Figure 3 Rate of dityrosine formation as a function of tyrosine concentration.

Assay of L-Tyrosine to Dityrosine Reaction

The parameters investigated in the reaction were the effects of horseradish peroxidase types I and

II, tyrosine and hydrogen peroxide concentration, and pH. The "standard" reaction conditions used were 0.7 m*M* hydrogen peroxide, 2.7 m*M* tyrosine, 1.6×10^{-7} *M* horseradish peroxidase, and 0.05*M* phosphate buffer. The reaction was carried out at 37°C and the rate of dityrosine formation was followed at 315 nm. The enzyme and hydrogen peroxide concentrations were determined at 403 nm[12] and 230 nm,[13] respectively. The reaction products were characterized by paper chromatography. Figures 2 and 3 show the rate of dityrosine formation from L-tyrosine as functions of peroxidase and tyrosine concentrations. Type II peroxidase had a dityrosine formation rate about twice that of the type I enzyme. The initial rates increased almost directly as the reactant concentrations indicated an approximate first-order reaction with respect to both enzyme and tyrosine concentration. This would suggest that Michaelis–Menten kinetics approximate the reaction process particularly with the type-II peroxidase, where presumably the Michaelis–Menten constant was much larger in value than the substrate concentration.

The effect of pH on dityrosine formation, shown in Figure 4, exhibited a maximum rate at pH = 8.0. Figure 5 presents the effect of hydrogen peroxide concentration on the reaction, showing that the rate of dityrosine production maximized at a peroxide concentration of 0.7 m*M*. The results were in general agreement with the findings of Bayse and coworkers,[14] except that the optimum pH was given as 8.2. The inhibitory effect of excess hydrogen peroxide also has been observed by Tenuovo and Paunio.[15]

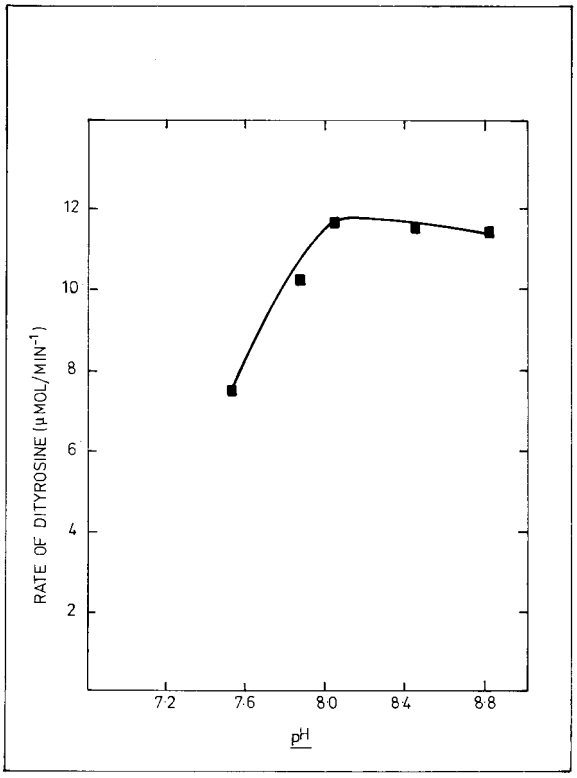

Figure 4 Rate of dityrosine formation as a function of pH.

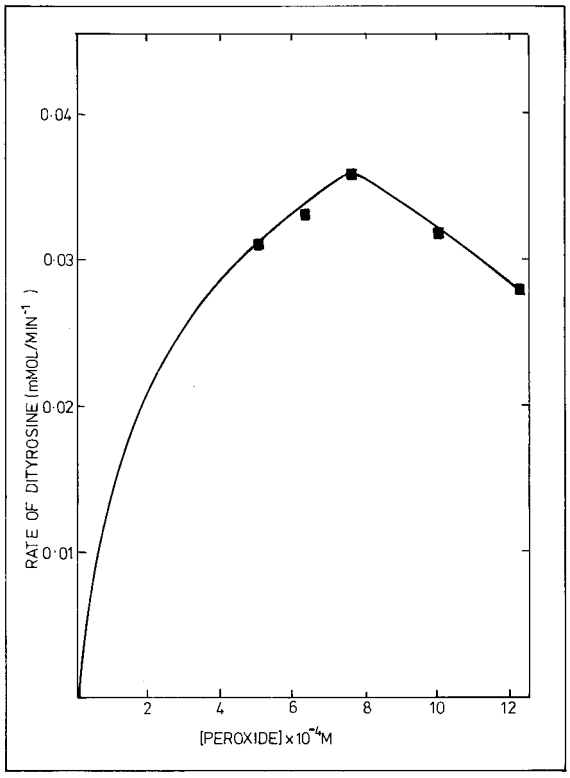

Figure 5 The effect of hydrogen peroxide on the rate of dityrosine formation.

However, the optimum conditions for the preparation of dityrosine were set by other practical considerations. First, tyrosine is sparingly soluble at the optimum pH of 8.0 in which it predominates as a zwitterion. Therefore, a pH of 9.2 had to be used in order to maximize the tyrosine solubility, despite the fact that this was not the optimum.

Second, oxidation products were formed as the reaction time was increased, making subsequent isolation of dityrosine more difficult. Consequently, a preheating or incubation procedure was adopted. Finally, the purification step with gel filtration chromatography (Sephadex G-10) gave improved yields when compared to a silica gel column with an aqueous butanol/acetic acid solvent. The final yield obtained was about 9–10%

of highly purified dityrosine. This is similar to the highest range quoted by Malanik and Ledvina,[9] but for a product which was of a lower purity. Certainly, the 10% yield maximum attained in this work was an improvement on that previously reported or obtained by repeating the method of Malanik and Ledvina.[9]

CONCLUSIONS

The horseradish peroxidase–hydrogen peroxidation of tyrosine to dityrosine was studied and optimum conditions elucidated. The yield and purification of the crude product were improved by using the type II peroxidase, increasing the tyrosine solubility, preheating the tyrosine solution, and using activated carbon and gel filtration chromatography (Sephadex G-10). The resulting dityrosine product was shown to be of high purity and could be used as a reliable reference for later wool studies.

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