Preparation of Photodynamic Hydrolysates from Bovine Hemoglobin

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Porphyric peptides were prepared from an agricultural byproduct, bovine hemoglobin. Hemoglobin was hydrolyzed with alcalase. After removal of heme iron, the total hydrolysate was separated on gel filtration. It consisted of three fractions; the major one was separated by reversed-phase performance chromatography. FAB analysis indicated that protoporphyrin, hematoporphyrin, and hydroxyethylvinyl deuteroporphyrin were the major porphyrin constituents. These porphyrins were maintained in solution by peptidic hydrolysates. The hydrophilic and photosensitizing properties of porphyrin–peptide complexes were demonstrated. The water-solubilizing ability of hydrolysates was significant and allowed amphiphilic porphyrins to be obtained. These compounds could give applications in different fields: they could be employed as insecticides and antibacterial products or even could give, after further purification, application in photodynamic therapy.

Keywords: *Porphyric peptides; hemoglobin; photosensitizing properties; hemoglobin hydrolysate*

INTRODUCTION

For many years, protein hydrolysates have been widely used for various applications. Food ingredients and more recently nutritional therapy were the main investigated fields (Cuthbertson, 1950; Silk et al., 1985). Additional studies have been undertaken within the past few years about the stimulating effects on fermentation (Tchorbanov and Lazarova, 1988), immune-stimulating properties (Migliore-Samour and Jollès, 1988), and biological activities (Henriques et al., 1987; Fiat and Jollès, 1989). Most of these studies concerned holoprotein hydrolysates derived from such diverse sources such as soy, casein, wheat, and fish (Kilara, 1985).

In a preceding paper (Piot et al., 1988), we reported the preparation of a defined decolorized enzymatic hydrolysate from bovine hemoglobin, considered as an agricultural byproduct. Studies on the applications of this hydrolysate have been carried out in many areas, including cellular cultivation (Dive et al., 1989) or nutrition (Léké et al., 1990).

In this work, we used hemoglobin as a typical pigment–protein complex in which heme is a naturally occurring pigment. It is well-known that the structural and functional association of pigments with proteins is often essential for photobiological processes (Masinovsky et al., 1989). Several experiments on the photochemical properties of pigments that result from formation of complexes with proteins have been performed (Shibata et al., 1986). Among pigments, hemin was shown to be photochemically inactive, whereas hemoprotenoid (hemin associated with copolymers of amino acids) exhibited a low photochemical activity (Masinovsky et al., 1989). Furthermore, porphyrins and porphyrin-related structures played a large number of well-diversified roles in food technology (Adler, 1973) and photochemistry. Thus, photoactivated porphyrins could display bactericidal (Malik et al., 1990), insecticidal (Rebeiz, 1993), or antitumorous effects (Bonnett and Berenbaum, 1989).

The aim of the present work was to prepare peptidic hydrolysates containing hydrophobic porphyrins from bovine hemoglobin and to compare their photosensitizing properties with a widely used mixture of soluble photosensitizers derived from hematoporphyrin (HpD). These preparations could be interesting in the context of the research of new insecticidal and antibacterial products.

MATERIALS AND METHODS

Preparation of Hemoglobin. Bovine blood, collected from the slaughterhouse, was centrifuged at 4000*g* for 15 min, and following the removal of the plasma, the red blood cells were washed three times with 0.9% NaCl. Hemoglobin was released by hemolyzing the cells in distilled water (1 vol of cells/1 vol of water) at 4 °C. The resulting hemoglobin concentration was determined according to the cyanmethemoglobin method (Crosby et al., 1954).

Hemoglobin Hydrolysis. Hydrolysis of hemoglobin was performed with alcalase (Novo Industrie, A/S, Bagsvaerd, Denmark) at 50 °C and pH 8.0. Hemoglobin (200 mL; 7% w/v) was hydrolyzed by alcalase (24 AU/kg of substrate) for 5 h; pH was maintained at 8.0 with a pH-stat (Metrohm, Switzerland) by addition of 0.5 M NaOH. The soluble fraction of hemoglobin hydrolysate was obtained after centrifugation at 20000*g* for 40 min.

Removal of Iron. One hundred milliliters of hemoglobin hydrolysate (5%) was brought to pH 1 by addition of oxalic acid (12.5 g, 1 M). The temperature was maintained at 80 °C to solubilize most of the oxalic acid. Ascorbic acid (1 g/L) was added to the solution to reduce the iron. The mixture was heated to boiling in a round-bottom flask equipped with a stirrer and a reflux condenser. Refluxing was continued for 30 min. The mixture was allowed to cool and then centrifuged. The filtrate was brought to pH 7.0 by addition of 1 M $Ca(OH)_2$. Calcium oxalate precipitate was discarded by centrifugation, and the supernatant containing porphyric peptides was freeze-dried.

The yield was calculated from the ratio of the heme amount in the initial solution to the amount of porphyrins recovered in the final product.

Heme content was determined according to the pyridine hemochromogen method (Paul et al., 1953). Porphyrin content was evaluated spectrophotometrically by reference to known concentrations of hematoporphyrin derivative prepared using

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the method of Lipson et al. (1961). Iron content was estimated by absorption spectrometry. The nitrogen content was determined according to the method of Kjeldahl (Gerhardt apparatus Vapodest 3). Protein content was expressed as N × 6.25.

UV–Vis Spectra. The UV–vis spectra of porphyric peptides were obtained with a spectrophotometer (Perkin-Elmer, France) in a phosphate buffer, pH 7.0. They were compared with those obtained from hematoporphyrin IX (HP), protoporphyrin IX (PP) (Sigma, France) in acetone/water, and hematoporphyrin derivative (HpD) in phosphate buffer, pH 7.0.

Gel Chromatography. Porphyric peptides (250 mg) were dissolved in 3 mL of 0.01 M borate buffer, pH 8.6, and chromatographed on a 90 × 5 cm column of Sephadex G25 SF (Pharmacia, France) with a 0.01 M borate buffer, pH 8.6. The flow rate was 40 mL/h, and the chromatographic profiles were recorded at 400 nm, using a Model 2151 LKB detector (LKB, Sweden). The void volume was measured using bovine serum albumin.

High-Performance Liquid Chromatography (HPLC). A Model 600 E delivery system, Model U 6K Universal injector, Model 445 UV detector, Model 2151 LKB vis detector, Model 745 integrator (Waters, France), and Delta-pak C_{18} 100-Å reversed-phase column (30 × 1.9 cm) were used.

Samples were eluted using 10 mM ammonium acetate buffer, pH 6.0, as eluent A and acetonitrile as eluent B. The gradient applied was 0–15% B in 15 min, 15–30% B in 30 min, and 30–100% B in 20 min. The flow rate was 10 mL/min. Samples were dissolved in eluent A (300 mg/mL), filtered on 0.45-μm filters (Millipore, France). Several injections of 100 μL were made, and absorbances were measured at 215 and 400 nm.

Mass Spectrometry Analysis of Porphyrins. Mass spectra generated from FAB-MS of porphyrins were recorded on a four-sector Concept II mass spectrometer (Kratos, U.K.) as previously described (Piot et al., 1992).

Partition Experiments. Fifty microliters of a concentrated solution of porphyric peptides or HpD (0.5 mg/mL) was added under stirring to a 2-mL solution of various HCl solutions (0.01–2.5% w/v). A 1-mL aliquot was taken to determine the absorption spectrum. The remaining volume was shaken for 3 min with an equal volume of solvent (ether or methyl ethyl ketone). The solution was centrifuged to ensure good separation of the two phases. Spectra of the aqueous and organic layer were recorded.

The results were expressed as percentages of porphyrins extracted by the organic solvent. Porphyrin content was determined spectrophotometrically from a standard curve established with HpD.

Photosensitizing Activity Determination. A specially designed thermostated cell (Kraayenhof et al., 1982) equipped with a Clark-type electrode (WTW, Germany) containing sensitizer (2×10^{-5} M) and 2×10^{-3} M histidine hydrochloride (chemical quencher of $^1O_2$) in phosphate buffer, pH 7.4 (1.6% NaCl buffered by 0.01 M NaH_2PO_4–Na_2HPO_4), was employed. The following sensitizers were used: HpD (prepared by the Nantes University Hospital pharmacy), heme peptides, or porphyrinic peptides (obtained by the procedure previously described). The cell was maintained at 30 °C and stirred at 600 rpm. Irradiations were performed with a Model GLG 5730 He–Ne laser (25 mW) (Nec Electronics, France) at 632.8 nm. Oxygen depletion during irradiation was followed by recording p_{O_2} measurements.

RESULTS AND DISCUSSION

Preparation. Hemoglobin was hydrolyzed by alcalase. Hydrolysis was followed by titration with 0.5 M NaOH utilizing a pH-stat (Figure 1). Hydrolysis was fast during the first 2 h and then became slower. The enzymatic hydrolysis was performed for 6 h and gave a heme peptidic hydrolysate.

The method of preparation of porphyric peptidic hydrolysate is illustrated in Figure 2. The amounts of iron, heme, porphyrins, and peptides recovered after each step are indicated. Iron was removed from the heme peptidic hydrolysate by complexation with oxalic acid. Most of the remaining undissolved heme was eliminated in the acidic precipitate. The yield of demetalation during the first step was about 35%. The neutralization of acidic supernatant to pH 7.0 (physiological condition) led to a decrease in the final yield (23%). Most of the peptides were recovered in the final neutral supernatant.

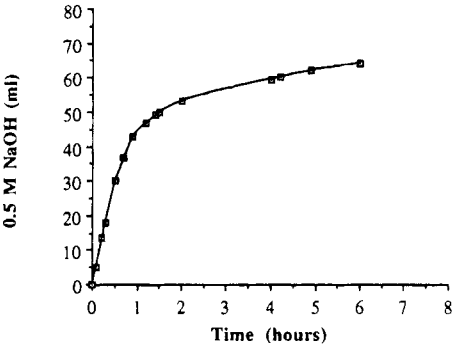
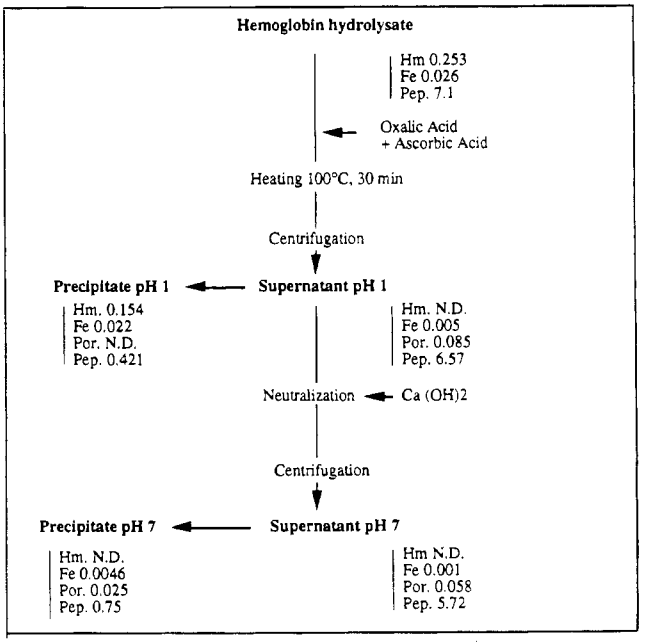


Figure 1. Kinetics of alcalase hemoglobin hydrolysis at pH 8 and 50 °C. Concentration of hemoglobin was 7% (w/v) and enzyme to protein ratio was 24 AU/kg of substrate. Hydrolysis was performed during 6 h.



N.D. : not detectable
Hm. : Heme, Fe : iron, Por. : porphyrins, Pep : peptides

Figure 2. Preparation of porphyric peptidic hydrolysates from bovine hemoglobin.

Porphyric peptides were fractionated by gel filtration chromatography (Figure 3). Thus, three porphyric peptidic fractions were eluted from the column: a major one and two smaller ones at 617, 937, and 1089 mL, respectively. In regard to the calibration curve (not shown), molecular masses of the peptides in fraction 1 could be estimated at greater than 5000 Da.

The porphyrin content in fraction 1 was 1.29% (w/v); the porphyrin contents were 0.115 and 0.193% (w/v) for fractions 2 and 3, respectively. Therefore particular attention was paid to the enriched fraction 1.

Characterization. The absorption spectrum of fraction 1 was performed in phosphate buffer, pH 7.0 (Figure 4). A large absorbance in the Soret region (400 nm) and four well-resolved bands with maxima at 503,

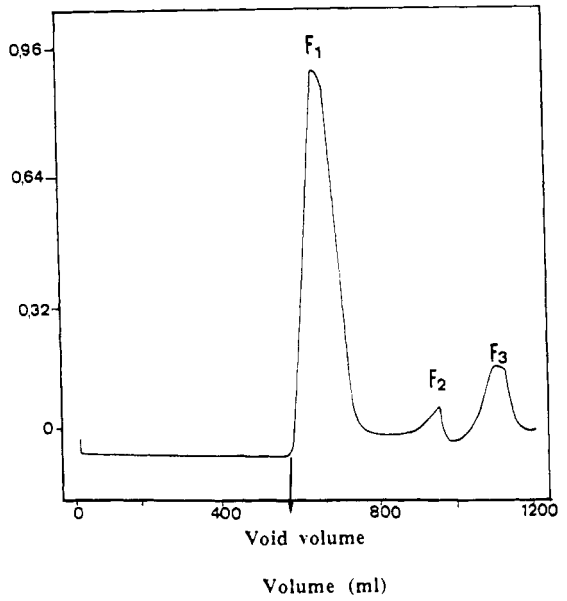


Figure 3. Fractionation of porphyric hydrolysates by gel filtration. Conditions: column, Sephadex G25 SF (90 × 5 cm); 10 mM borate buffer, pH 8.6; flow rate 50 mL/h; detection, 400 nm; injection, 250 mg/3 mL.

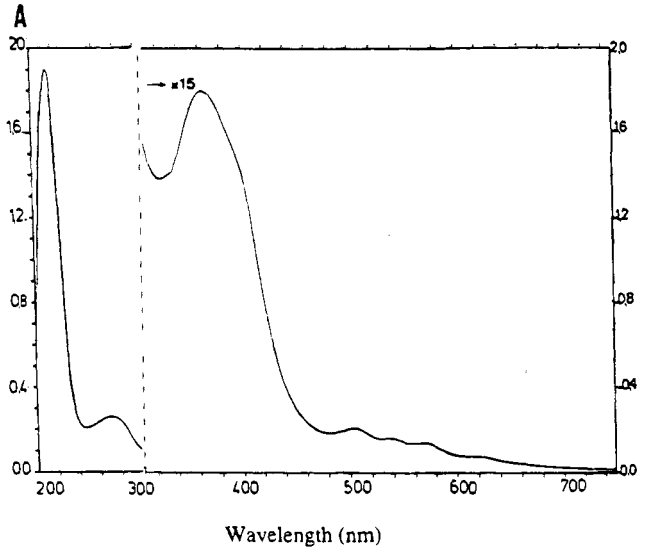


Figure 4. UV–vis spectrum of fraction 1. Conditions: solvent, phosphate buffer, pH 7; concentration of fraction 1 was 2.5 mg/mL.

540, 571, and 623 nm are characteristic of porphyrins (Falk, 1963). Absorbance at 280 nm shows the presence of aromatic amino acids. The four visible bands are shifted to longer or smaller wavelengths compared with those of hematoporphyrin, protoporphyrin, and hematoporphyrin derivative (Table 1). Strong porphyrin–peptide associations could explain this phenomenon.

To study the nature of the porphyrin–peptide associations in fraction 1, further purification of this fraction was attempted by reversed-phase HPLC (Figure 5). We used the gradient conditions described under Materials and Methods. It appeared that porphyrins and peptides were individually eluted, demonstrating the absence of covalent bonds between them. The hydrophilic peptides were eluted first, while the porphyrins, more hydrophobic, were eluted with acetonitrile concentration between 50 and 100%.

The two most important fractions (1α and 1β) were collected, and FAB mass spectrometry was used to

Table 1. Absorption Characteristics of Fraction 1 and Known Porphyrins

		absorption maxima (nm)				
sample	buffer	Soret band	I	II	III	IV
protoporphyrin	phosphate buffer, pH 7/acetone	400	502	536	574	627
hematoporphyrin	phosphate buffer, pH 7/acetone	400	494	528	565	618
HpD	phosphate buffer, pH 7	372	505	538	567	618
fraction 1	phosphate buffer, pH 7	364	503	540	571	623

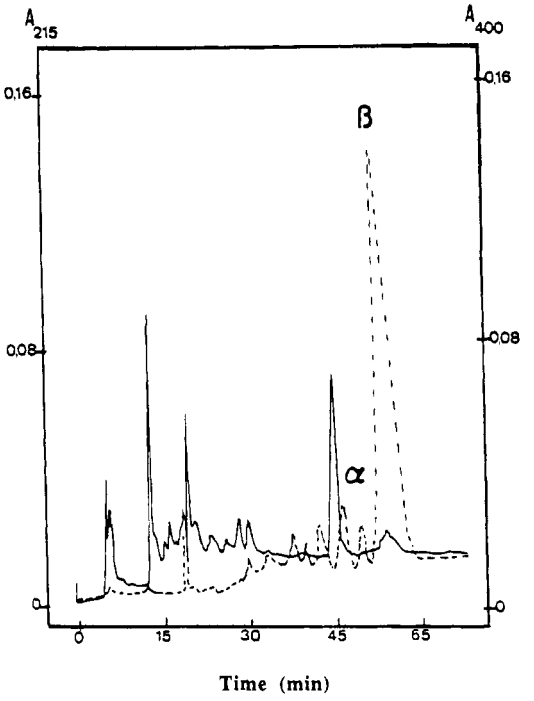


Figure 5. HPLC separation of porphyric peptides. Conditions: C_{18} 100-Å Delta-pak column; solvent A, 10 mM ammonium acetate, pH 6.0; solvent B, 100% acetonitrile; gradient, 0–15% solvent B in 15 min, 15–30% solvent B in 30 min, 30–100% B in 20 min; flow rate, 10 mL/min; detection at 215 and 400 nm.

characterize the porphyrins of fractions 1α and 1β. The FAB mass spectrum of fraction 1α shows three major peaks at *m/z* 595.3, 581.2, and 564.5. These peaks most likely correspond to hematoporphyrin (*m/z* 595.3) and to the species formed by dehydration of the secondary alcohol substituents of this porphyrin, i.e., hydroxyethylvinyl deuteroporphyrin (*m/z* 581.2) and protoporphyrin (*m/z* 564.5), since dehydration of secondary alcohols in acidic solvents is a well-known process (Van Berkel et al., 1991). Further mass spectrometry studies showed that fraction 1β contained dehydrated monomers (*m/z* 580.5 and 564.6) corresponding to [HP − $(H_2O)_n$], i.e., hydroxyethylvinyl deuteroporphyrin (n = 1) and protoporphyrin (n = 2). This fraction contains also dimers of hydroxyethylvinyl deuteroporphyrin (*m/z* 1161.3). Fraction 1β comprised dehydrated higher molecular weight oligomers (dimers to tetramers) characterized by the loss of water molecules (*m/z* 1145.3, 1724.2, 2304.7) or n[HP − H_2O] − H_2O as previously described (Musselman et al., 1988). The FAB results confirm the absence of porphyrin–peptide covalent bonds in fraction 1.

Water Solubility. Additional experiments were undertaken to determine hydrophilic properties of por-

Table 2. Partition of Porphyric Peptides and Hematoporphyrin Derivative between Ether and HCl Aqueous Solution from 0.01 to 2.5% (w/v) (Porphyrin Concentration Was 0.5 mg/mL)

		HCl				
sample		0.01%	0.05%	0.1%	0.22%	2.5%
fraction 1	porphyrin in ether (%)	0	0	0	0	0
HpD	porphyrin in ether (%)	85	31	25	11	0

phyric peptides of fraction 1. Thus, the partition between ether and aqueous solutions was studied for this fraction 1 in comparison with HpD, the most employed water-soluble porphyric photosensitizers (Table 2). We characterized the hydrophilic properties of porphyric peptides and HpD according to the original method of Willstätter and Mieg (Zeile and Rau, 1937). This method defines the "HCl number" as the concentration of HCl in percent (w/v) which is, from an equal volume of an ether solution of the porphyrin, capable of extracting two-thirds of the porphyrin to the aqueous solution. The HCl number found for HpD is about 0.05% (Table 2), a value close to the one reported in the literature for hematoporphyrin (Smith, 1975).

More surprising was the behavior of porphyric peptides which were completely soluble in aqueous solution and not extractable by ether. In this case, it is not possible to determine the HCl number. It may be concluded from these results that this fraction was particularly hydrophilic. In spite of the absence of covalent bonds between porphyrins and peptides, the weak noncovalent association may be important enough to prevent extraction of the hydrophobic porphyrins toward the organic layer.

Another experiment was carried out at pH 2 with methyl ethyl ketone, which usually completely separates heme from protein in hemoproteins (Teale, 1959). The percentage of porphyrin extraction into methyl ethyl ketone for HpD was 89%, vs 0% for porphyric peptides. This confirms the results obtained with ether and corroborates the hydrophilic character of the porphyric fraction. One may speculate about the importance of the peptidic environment of porphyrin in this product.

Photosensitizing Properties. A system to study the photosensitizing properties of porphyric peptides was designed according to the method of Gottfried et al. (1988). Upon irradiation with a laser (632.8 nm), energy is transferred from photosensitizer to oxygen, which is converted to the singlet state and then reacts with histidine (a singlet oxygen acceptor).

When an aqueous solution of fraction 1 was irradiated, we observed a decrease in O_2 concentration (Figure 6). Under the same conditions HpD gave a slightly more pronounced O_2 concentration depletion. Heme peptides or buffer alone gave a weak oxygen depletion due to oxygen consumption by Clark-type electrode. This confirms the photosensitizing activity of the porphyric peptides which were prepared from the heme peptidic hydrolysate. The relative activity of fraction 1 in comparison with HpD, calculated from the slope in Figure 6, is about 75%. This value demonstrates that porphyric peptides could be interesting potential photosensitizers.

Conclusion. In this paper, the preparation of porphyric peptides from an agricultural waste, slaughterhouse bovine hemoglobin, is described. The main results can be summarized as follows: A fraction has been obtained by gel filtration chromatography; this

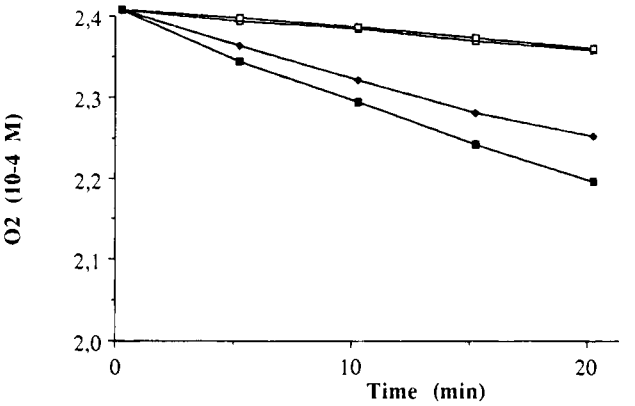


Figure 6. Photosensitizing activity measurements. The oxygen consumption was followed by irradiation at 632.8 nm (laser He–Ne) of porphyric samples (2×10^{-5} M) at 37 °C in a phosphate buffer, pH 7.4. (⊡) Heme peptides; (□) buffer; (◆) fraction 1; (■) hematoporphyrin derivative.

fraction is characterized by particular hydrophilic properties in comparison with HpD. It is a mixture of hydrophobic porphyrins maintained in aqueous solution with amphiphilic peptides. The nature of the porphyrins contained in this fraction was characterized by UV–vis spectroscopy and chromatographic behavior. A system was developed to estimate the photosensitizing properties of fraction 1 in comparison with HpD. This fraction seems quite promising for further applications based on photosensitization: porphyric insecticide technology, photobiotechnology, and photodynamic therapy.

Future studies to determine the oxygen quantum yield of the porphyrin peptides in fraction 1 and its photodynamic effect on cultured tumor cells is warranted to quantify the photosensitizing properties of these complexes. These results will be dealt with in a future paper. Moreover, further purifications of porphyric peptides will be undertaken to better characterize the porphyric peptide fraction.

LITERATURE CITED

Adler, A. D. The chemical and physical behaviour of porphyrins compounds and related structures. *Ann. N. Y. Acad. Sci.* **1973**, *206*, 7–17.

Bonnett, R.; Berenbaum, M. Porphyrins as photosensitizers. *Ciba Found. Symp.* **1989**, *146*, 40–59.

Crosby, W. H.; Munn, J. I.; Furth, F. W. Standardizing a method for clinical hemoglobinometry. *U.S. Armed Forces Med. J.* **1954**, *5*, 693–703.

Cuthbertson, D. P. Amino-acids and proteins hydrolysates in human and animal nutrition. *J. Sci. Food Agric.* **1950**, *1*, 35–41.

Dive, D.; Piot, J. M.; Sannier, F.; Guillochon, D.; Chavet, P.; Lutrat, S. Use of hemoglobin enzymic hydrolysates prepared on a pilot-plant scale as a nitrogen source for the cultivation of three species of Tetrahymena. *Enzyme Microb. Technol.* **1989**, *11*, 165–169.

Falk, E. Chemistry and biochemistry of porphyrins and metalloporphyrins. In *Comprehensive Biochemistry*; Florkin, M., Stotz, E. H., Eds.; Elsevier: Amsterdam, 1963; Vol. 9, pp 3–31.

Fiat, A. M.; Jollès, P. Casein of various origins and biologically active casein peptides and oligosaccharides: structural and physiological aspects. *Mol. Cell. Biochem.* **1989**, *87*, 5–30.

Gottfried, V.; Peled, D.; Winkelman, J. W.; Kimel, S. Photosensitizers in organized media: singlet oxygen production and spectral properties. *Photochem. Photobiol.* **1988**, *48*, 157–163.

Henriques, O. B.; De Deus, R. B.; Santos, R. A. S. Bradykinin potentiating peptides isolated from α-casein tryptic hydrolysate. *Biochem. Pharmacol.* **1987**, *36*, 182–184.

Kilara, A. Enzyme-modified food ingredients. *Process Biochem.* **1985**, *21*, 149–157.

Kraayenhof, R.; Schuurmans, J. J.; Valkier, L. J.; Veen, J. P. C.; Marum, D. V.; Jasper, C. G. G. A thermoelectrically regulated multipurpose cuvette for simultaneous time-dependent measurements. *Anal. Biochem.* **1982**, *127*, 93–99.

Léké, A.; Piot, J. M.; Guillochon, D.; Ricard, J.; Postel, J. P.; Risbourg, J. P.; Canarelli, J.P. Comparative absorption of two protein hydrolysates (bovine hemoglobin and casein) in the intestine of the conscious pig. *Nutr. Clin. Metabol.* **1990**, *4*, 223–229.

Lipson, R. L.; Baldes, E. J.; Olsen, A. M. The use of a derivative of haematoporphyrin in tumor detection. *J. Natl. Cancer Inst.* **1961**, *26*, 1–11

Malik, Z.; Hanania, J.; Nitzan, Y. Bactericidal effects of photoactivated porphyrins. An alternative approach to antimicrobial drugs. *J. Photochem. Photobiol. B: Biol.* **1990**, *5*, 281–293.

Masinovsky, Z.; Lozovaya, G. I.; Sivash, A. A.; Drasner, M. Porphyrin-proteinoid complexes as models of prebiotic photosensitizers. *BioSystems* **1989**, *22*, 305–310.

Migliore-Samour, D.; Jollès, P. Casein, a prohormone with an immunomodulating role for the newborn. *Experientia* **1988**, *44*, 188–193.

Musselman, B.; Kessel, D.; Chang, C. K. Fast atom bombardment mass spectrometry of high molecular weight fraction of porphyrin-based photodynamic therapy drugs. *Biomed. Environ. Mass Spectrom.* **1988**, *15*, 257–263.

Paul, K. G.; Theorell, H.; Akeson, A. The molar light absorption of pyridine ferroprotoporphyrin (pyridine heamochromogen). *Acta Chem. Scand.* **1953**, *7*, 1284–1287.

Piot, J. M.; Guillochon, D.; Leconte, D.; Thomas, D. Application of ultrafiltration to the preparation of defined hydrolysates of bovine hemoglobin. *J. Chem. Technol. Biotechnol.* **1988**, *42*, 147–156.

Piot, J. M.; Zhao, Q.; Guillochon, D.; Ricard, G.; Thomas, D. Isolation and characterization of a bradykinin peptide from a bovine peptic hemoglobin hydrolysate. *Biochem. Biophys. Res. Commun.* **1992**, *189*, 101–110.

Rebeiz, C. A. Porphyric insecticides. *J. Photochem. Photobiol. B: Biol.* **1993**, *18*, 97–99.

Shibata, H.; Ochiai, H.; Kamashima, T.; Okamoto,T.; Inamura, I. Preparation and properties of the water soluble chlorophyll-bovine serum albumin complexes. *Biochim. Biophys. Acta* **1986**, *852*, 175–182.

Silk, D. B. A.; Grimble, G. K.; Rees, R. G. Protein digestion and amino-acid and peptide absorption. *Proc. Nutr. Soc.* **1985**, *44*, 63–72.

Smith, K. M. General features of the structure and chemistry of porphyrins compounds. In *Porphyrins and Metalloporphyrins*; Smith, K. M., Ed.; Elsevier: Amsterdam, 1975; Vol. I, pp 3–27.

Tchorbanov, B.; Lazarova, G. Evaluation of protein hydrolysate using the fermentation activity of immobilized cells. *Biotechnol. Appl. Biochem.* **1988**, *10*, 301–304.

Teale, F. W. J. Cleavage of the haem-protein link by acid methylethylketone. *Biochim. Biophys. Acta* **1959**, *35*, 543.

Van Berkel, G. J.; McLuckey, S. A.; Glish, G. L. Electrospray ionization of porphyrins using a quadrupole ion trap for mass analysis. *Anal. Chem.* **1991**, *63*, 1098–1109.

Zeile, K.; Rau, B. Partition of porphyrins between ether and salted solutions. *Z. Physiol. Chem.* **1937**, *250*, 197–217.

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