3. A. K. Ivanov, E. É. Lavut, A. A. Antonov, and V. N. Karel'skii, Khim. Prir. Soedin., No. 5, 684 (1989).
4. L. F. Johnson, I. L. Schwartz, and R. Walter, Proc. Natl. Acad. Sci. USA, 64, 1269 (1969).

SYNTHESIS AND PROPERTIES OF PEPTIDE FRAGMENTS OF THE S-REGION OF THE SURFACE PROTEIN OF THE HEPTATITIS B VIRUS

I. L. Kuranova, S. I. Churkina, A. V. Os'mak, E. B. Filonova, V. L. Lyudmirova, and O. V. Noskova

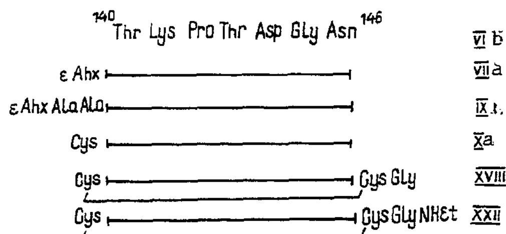
UDC 547.4.057

A peptide fragment with the 140–146 sequence of the main component of the surrace protein of the hepatitis B virus (HBsAG) and a number of its structural analogues have been synthesized by the classical method in solution. Conjugates of the peptides synthesized with bovine serum albumin and with a synthetic polypeptide analogue of polytuftsin have been obtained. The ability of the preparations to bind antibodies from the blood sera of hepatitis B patients has been studied. The possibility has been shown of their use for revealing antibodies to the heptatitis B virus in solid-phase enzyme-mediated immunoassay.

According to the WHO, viral hepatitis B is one of the most acute problems of public health [1]. The extremely widespread nature of this disease is connected with the absence of an effective vaccine and of a reliable system of diagnosis. And, if it is borne in mind that, because of the absence of effective systems for the replication of the virus, the main source of viral proteins still remains donor blood, the interest aroused by investigations on the synthesis of the antigenic determinants of the hepatitis B virus capable of replacing the natural material in the vaccines used and in diagnostic systems becomes understandable.

The surface antigen of the hepatitis B virus, which possesses a high antigenic and immunogenic activity, is a complex of three glycosylated proteins containing a common sequence of 226 amino acid residues (the S-protein). In addition to the main S-protein, which is the principal marker of the disease, the outer coat of the virus includes two minor proteins: medium (S+preS$_2$) and major (S+preS$_2$+preS$_1$).

With the aim of studying the structural-functional organization and immunochemical properties of the antigenic determinants and also of creating an effective test system for the hepatitis B virus, we have carried out the synthesis of a fragment with the sequence 140–146 of the main surface protein (VIb), which is part of the conformation-dependent group-specific determinant "a," common for all the subtypes of the virus [2, 3], and a number of its structural analogues:



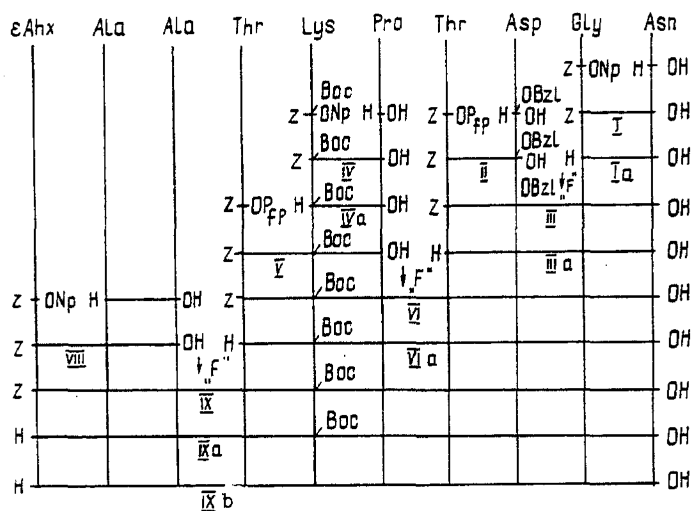
where εAhx represents 6-aminohexanoic acid. The εAhx was introduced into the peptide sequence [in peptides (VIIa) and (IXb)] as a spacer for the subsequent conjugation of the haptens with

Leningrad State University. Translated from Khimiya Prirodnykh Soedinenii, Nos. 3,4, pp. 406-413, May-August, 1992. Original article submitted August 19, 1991.

0009-3130/92/0304-0355$12.50 © 1992 Plenum Publishing Corporation

a high-molecular-mass carrier. In peptide (IXb) the cysteine in positions 138 and 139 of the native protein was replaced by alanine. In order to fix a conformation close to the natural one (presumable β-bend), an analogue of the 139–147 sequence (XVIII) was synthesized in which a disulfide ring had been closed between the cysteine residues [4] and glycine had been introduced as a spacer group in position 148. The peptide ethylamide (XXII) was obtained in order to improve sorbability on the polystyrene planchets used in EIA.

The synthesis of the peptides was carried out by the classical method in solution with a combination of the stepwise growth of the chain by the activated-ester method and the block condensation of fragments. To protect the α-amino function we used the benzyloxycarbonyl (Z) and tert-butoxycarbonyl (Boc) groups, which were eliminated by catalytic hydrogenolysis and acidolysis, respectively, while the ε-amino function of lysine was protected by a Boc group. The sulfhydryl function of cysteine was protected by an acetamidomethyl group the elimination of which with the formation of a cyclic compound was achieved by the action of iodine in methanol [5]. The β-carboxy group of aspartic acid was protected by the formation of benzyl (scheme 1) or tert-butyl (scheme 2) esters. All the peptides synthesized contained the combination Asp–Gly, prone to α → β-transpeptidization, which made it undesirable to use excesses of bases and acids in the process of synthesis [6]. At all the stages of acylation (apart from the formation of dipeptides) the amino component was introduced with a free carboxyl group, which excluded the risk of the presence of an excess of a base in the reaction mixture. The schemes of synthesis were selected in such a way that the acidolytic deblocking of the peptides was carried out in the final stage, so that the risk of obtaining transpeptidization products with the unnatural (β) linkage of these residues was diminished. IR spectroscopy confirmed the absence of an intermediate aminosuccinyl derivative, and HPLC the individuality of the peptides obtained.

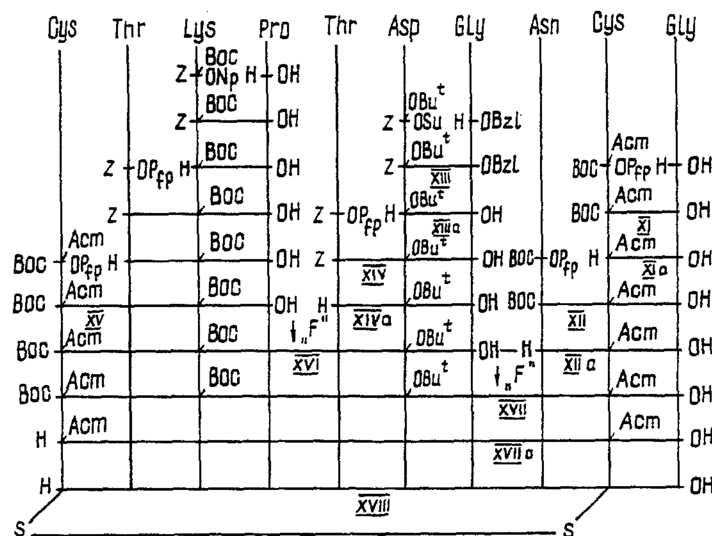


Scheme 1

To improve their immunogenic proeprties, peptides (VIb), (VIIa), and (IXb) were conjugated with succinylated bovine serum albumin (BSA) [7]. The degree of conjugation amounted to 9-12 molecules of peptide to one molecule of protein (determined from the results of amino acid analysis). Peptide (Xa) was conjugated with a synthetic carrier – a polytuftsin analogue (molecular mass 12-14 thousand) [8].

The peptides and their conjugates that had been obtained were investigated by the method of solid-phase enzyme-mediated immunoassay for their ability to bind with antibodies in samples of blood sera from hepatitis B patients. (See scheme 2 on following page.)

The antigenic determinants were fixed to the polystyrene planchets for EIA by two methods: through physical sorption and with the aid of covalent binding. It was found that the second method of immobilizing the peptides permitted an immunosorbent to be obtained that was distinguished by a higher sensitivity and specificity. The concentration of peptide sorbed on the polystyrene was important. A rise in the concentration of determinants to 10 μg/ml increased nonspecific sorption in the performance of the EIA reaction, which led to false-positive results.



Scheme 2

It was found that the cyclic peptide (XVIII) and a conjugate of peptide (IXb) with succinylated BSA permitted antibodies in subjects infected with hepatitis B to be detected in approximately 60% of cases. At the same time, samples of sera from hepatitis B patients revealed with the conjugate of the peptide (IXb) did not react with peptide (XVIII). In addition, it was shown that peptide (XVIII) and the peptide ethylamide (XXII) possessed a considerably greater specificity in the cyclic form that in the linear form, while the ethylamide (XXII) had a better sorption capacity than peptide (XVIII).

The results of the investigations showed that it is apparently necessary to use a combination of two or more antigenic determinants for the complete revelation of the antibodies in the blood sera of hepatitis B patients.

EXPERIMENTAL

We used L-amino acids for synthesis. Melting points were determined on a Kofler instrument and are given without correction. The individuality of the compounds obtained was checked with the aid of TLC on plates with a fixed layer of Kieselgel 60 F254 (Merck). The chromatographic mobilities given relate to the following systems: 1) butan-1-ol—acetic acid—water (3:1:1); 2) butan-1-ol—acetic acid—pyridine—water (30:6:20:24); 3) butan-1-ol—acetic acid—pyridine—water (10.5:1:6:7.5); and 4) ethyl acetate—pyridine—acetic acid—water (240:20:6:11). Electrophoretic mobilities were determined relative to histidine on FN-12 paper (Filtrak) at a potential gradient of 30V cm^{-1} in 2% acetic acid (pH 2.6). The amino acid analysis of the peptides was conducted on an AAA-T 339 analyzer (Microtechna, Prague). Elementary analysis was carried out on a Hewlett-Packard 185 C, H, N analyzer. Specific rotations were determined on a Perkin-Elmer 141 polarimeter. HPLC was conducted in an Altex 332 liquid chromatograph (USA): column 4 × 250 mm, LiChrosorb RP-18 (5 μm) (analytical variant); column 10 × 250 mm, Ultrasphere ODS (5 μm) (preparative variant). Ion-exchange chromatography was performed on SP-Sephadex C-25 in ammonium acetate buffer (pH 7.8) in a gradient regime in the range of molarities of the buffer of from 0.10 to 0.5 M (column 1.3 × 23 cm, rate of elution 30 ml/h, detection at λ 214 nm). As the detecting instrument we used a Single Path Monitor UV (LKB, Sweden). Column chromatography made use of silica gel L 40/100 (Chemapol, Czechoslovakia) in a 2.5 ×50 cm column; and Sephadex G-25 (Pharmacia, Sweden) in a 1.5 × 93 cm column. The elementary analyses of all the protected peptides corresponded to the calculated figures.

1. ZGlyAsnOH (I). A solution of 4.9 g (32.5 mmole) of asparagine in 32.5 ml of 1 N NaOH was treated with 11.8 g (35.1 mmole) of ZGlyONp in 20 ml of dioxane. The reaction mixture was kept for 48 h and then the dioxane was evaporated off, the residue was diluted with water and was extracted with ethyl acetate (3 × 20 ml), and the aqueous layer was acidified with 1 N H_2SO_4 and extracted with n-butanol (3 × 20 ml). The combined extract was washed with water and was evaporated. After reprecipitation from isopropanol with ether, 7.0 g (67%) of the dipeptide (I) was obtained, with mp 127-129°C, R_f 0.74 (system 1).

2. ZThrAsp(OBzl)OH (II). To 1.4 g (6.1 mmole) of β-benzyl aspartate in 3 ml of methanol was added 13.7 ml (6.1 mmole) of a 0.45 N methanolic solution of Triton B. The methanol was evaporated off, and the residue was dissolved in 3 ml of DMFA and was treated with 3.1 g (7.4 mmole) of ZThrOPfp. The reaction mixture ws stirred for 2 h and, after the addition of 10 ml of a 5% solution of $NaHCO_3$, it was extracted with ether (2 × 20 ml), and was then acidified with $KHSO_4$ to pH 3. The oil that had separated out was extracted with ethyl acetate (3 × 40 ml), and the combined extract was washed with water, dried with Na_2SO_4, and evaporated. This gave 2.4 g (86%) of substance (II) in the form of a dry foam, R_f 0.33 (system 4).

3. ZThrAsp(OBzl)GlyAsnOH (III). Dipeptide (I) (0.7 g; 2.0 mmole) was hydrogenated in 30 ml of 70% aqueous methanol over a Pd/C catalyst. After 2 h, the catalyst was filtered off, the filtrate was evaporated, and the residue was crystallized from isopropanol. This gave 0.4 g (92%) of substance (Ia), R_f 0.60 (system 1), E_{His} 0.74.

A solution of 0.7 g (1.5 mmole) of the dipeptide (II) in 10 ml of ethyl acetate was treated with 1.3 g (1.7 mmole) of complex F. The reaction mixture was stirred at room temperature for 30 min and at 0°C for 1 h. Then the DCHU was filtered off, and the filtrate was evaporated. The residue was dissolved in 3 ml of DMFA and this solution was added to a solution of 0.34 g (1.78 mmole) of substance (Ia) in DMFA. The reaction mixture was stirred for 12 h, the solvent was evaporated off, and the residue was crystallized from ethyl acetate. This gave 0.8 g (83%) of the tetrapeptide (III), mp 108-110°C, R_f 0.64 (system 1), 0.68 (system 3). Amino acid analysis: Thr 0.88 (1), Asp 2.10 (2), Gly 1.00 (1).

4. ZLys(Boc)ProOH (IV) was obtained form 1.1 g (9.5 mmole) of proline and 4.0 g (8.0 mmole) of ZLys(Boc)ONp under the conditions of reaction 2. In this way, 2.9 g (74%) of dipeptide (IV) was isolated in the form of a dry foam, R_f 0.60 (system 4).

5. ZThrLys(Boc)ProOH (V). Dipeptide (IV) (0.8 g; 1.7 mmole) was hydrogenated in 30 ml of methanol over a Pd/C catalyst. This gave 0.5 g (92%) of product (IVa), R_f 0.30 (system 1), E_{His} 0.71.

To 0.3 g (0.9 mmole) of dipeptide (IVa) in 3 ml of DMFA was added 0.4 g (1.0 mmole) of ZThrOPfp. After 10 h the reaction product was precipitated with ether. The substance was chromatographed on a column of silica gel L 40/100 in the ethanol—ethyl acetate (1:3) system. This led to the isolation of 0.4 g (82%) of the tripeptide (V) in the form of a dry foam, R_f 0.29 (system 4).

6. ZThrLys(Boc)ProThrAspGlyAsnOH (VI). The tetrapeptide (III) (0.4 g; 0.6 mmole) was deblocked by the method of 3, which gave 0.2 g (92%) of product (IIIa), R_f 0.46 (system 2), E_{His} 0.69.

Tripeptide (V) (0.5 g; 0.8 mmole) in 10 ml of ethyl acetate was treated with complex F (0.7 g; 0.9 mmole). The reaction mixture was kept at room temperature for 30 minutes and at 0°C for 1 h, the DCHU was filtered off, the filtrate was evaporated, the residue was dissolved in 3 ml of DMFA, and this solution was added to 0.2 g (0.5 mmole) of tripeptide (IIIa) in 3 ml of DMFA. The reaction was conducted for 12 h, and the reaction mixture was worked up by the method of paragraph 3. This gave 0.4 g (86%) of the heptapeptide (VI), mp 108-110°C, R_f 0.50 (system 1), 0.55 (system 3).

7. ThrLys(Boc)ProThrAspGlyAsnOH (VIa). Peptide (VIa) (0.4 g; 0.4 mmole) was deblocked by the method of paragraph 3. This gave 0.3 g (87%) of a substance which was purified on a column of DEAE-Sephadex A-25 in a gradient of ammonium acetate buffer. In this way, 0.209 g of the heptapeptide (VIa) was isolated. Amino acid analysis: Thr 2.30 (2), Lys 1.10 (1), Pro 0.98 1), Asp 2.10 (2), Gly 1.00 (1).

8. ThrLysProThrAspGlyAsnOH (VIb). Substance (VIa) (0.05 g; 0.03 mmole) was deblocked by the action of 0.3 ml of a 2 N solution of HCl in dioxane for 50 min. The reaction product was precipitated with ether, giving 0.04 g (90%) of a substance with R_f 0.30 (system 2), 0.44 (system [illegible]); E_{His} 0.58.

9. εAhxThrLys(Boc)ProThrAspGlyAsnOH (VII) was obtained from 0.10 g (0.12 mmole) of the heptapeptide (VIa) and 0.063 g (0.16 mmole) of ZεAhxONp in 3 ml of DMFA. After 12 h, the reaction mixture was precipitated with ethyl acetate. The substance obtained was hydrogenated in 10 ml of 90% aqueous methanol over a Pd/C catalyst, and the hydrogenation product was purified with the aid of ion-exchange chromatography under the conditions of experiment 7. This gave 0.068 g (60%) of a substance with R_f 0.42 (system 2), 0.33 (system 3); E_{His} 0.56.

10. εAhxThrLysProThrAspGlyAsnOH (VIIa). Substance (VII) (0.030 g; 0.016 mmole) was deblocked by the action of 0.2 ml of a 2 N solution of HCl in dioxane. This gave 0.029 g (98%) of a substance with R_f 0.37 (system 2), 0.30 (system 3); E_{His} 0.61.

11. ZεAhxAlaAlaOH (VIII) was obtained from 0.6 g (3.9 mmole) of AlaAlaOH and 1.2 g (3 mmole) of ZεAhx in a way similar to that of experiment 1. The yield of product was 1.0 g (79%), mp 131-133°C, R_f 0.80 (system 1).

12. ZεAhxAlaAlaThrLys(Boc)ProThrAspGlyAsnOH (IX). A solution of 0.10 g (0.12 mmole) of the heptapeptide (VIa) in 3 ml of DMFA was treated with the ZεAhxAlaAlaOPfp obtained from 0.066 g (0.15 mmole) of peptide (VIII) and 0.125 g of complex F in ethyl acetate. After 12 h the product was precipitated with ethyl acetate. This gave 0.10 g (91%) of a substance with mp 106-109°C, R_f 0.48 (system 1), 0.52 (system 3).

13. εAhxAlaAlaThrLys(Boc)ProThrAspGlyAsnOH (IXa). Substance (IX) 0.10 g; 0.09 mmole) was hydrogenated in 10 ml of aqueous methanol over a Pd/C catalyst. The product was purified on a column of DEAE-Sephadex A-25 in an ammonium acetate buffer. This gave 0.052 g (60%) of a substance with R_f 0.28 (system 2), 0.17 (system 3); E_{His} 0.45.

14. εAhxAlaAlaThrLysProThrAspGlyAsnOH (IXb). Peptide (IXa) (0.020 g; 0.019 mmole) was deblocked by the action of 0.2 ml of a 2 N solution of HCl in dioxane. The yield of product was 0.018 g (quantitative), R_f 0.25 (system 2); E_{His} 0.52.

15. BocCys(Acm)ThrLys(Boc)ProThrAspGlyAsnOH (X). A solution of 0.21 g (0.25 mmole) of the heptapeptide (VIa) in 3 ml of DMFA was treated with 0.17 g (0.38 mmole) of BocCys-(Acm)OPfp. After 12 h the reaction product was precipitated with ethyl acetate and dissolved in 50 ml of n-butanol, and the solution was washed with water (4 × 5 ml). The solvent was evaporated off, to give 0.23 g (84%) of the octapeptide (X), mp 114-118°C, R_f 0.24 (system 1), 0.42 (system 3).

16. Cys(Acm)ThrLysProThrAspGlyAsnOH (Xa). The octapeptide (X) (0.2 g; 0.18 mmole) was deblocked by the action of 0.75 ml of a 2 N solution of HCl in dioxane in the presence of dimethyl sulfide for 50 min. The reaction product was precipitated with ether. This gave 0.18 g of a substance which was purified on a column of DEAE-Sephadex A-25 in an ammonium acetate buffer with an ionic strength gradient of from 0.01 to 0.5 M. After lyophilization, 10 g (40%) of peptide (Xa) was obtained, with R_f 0.12 (system 2), 0.38 (system 3); E_{His} 0.79. Amino acid analysis: Asp 1.82 (2), Thr 2.03 (2), Pro 1.07 (1), Gly 0.96 (1), Cys 0.62 (1) (decomp.), Lys 1.00 (1).

17. BocCys(Acm)GlyOH (XI) was obtained from 0.3 g (4.1 mmole) of glycine and 2.2 g (4.6 mmole), of BocCys(Acm)OPfp by a method similar to that of paragraph 1, with a yield of 1.3 g after recrystallization from propan-2-ol–ether, mp 140-143°c, R_f 0.79 (system 1), 0.52 (system 4).

18. BocAsnCys(Acm)GlyOH (XII). The dipeptide (XI) (1.3 g; 3.5 mmole) was deblocked as in experiment 16. The hydrochloride so obtained was dissolved in 3 ml of DMFA, and 0.44 ml (3.5 mmole) of NEM and 1.5 g (3.8 mmole) of BocAsnOPfp were added. The reaction mixture was allowed to stand for 24 h and was then worked up as in experiment 3. After recrystallization from isopropanol–ethyl acetate, 1.1 g (77%) of tripeptide (XII) was obtained with mp 126-129°c, R_f 0.57 (system 1).

19. ZAsp(OBu^t)GlyOBzl (XIII). A solution of 0.8 g (5.0 mmole) of the benzyl ester of glycine in 5 ml of DMFA was treated with 1.6 g (3.8 mmole) of ZAsp(OBu^t)OSu. The reaction mixture was left for 4 h, the solvent was evaporated off, the residue was dissolved in ethyl acetate, and the solution was washed with water. Evaporation of the solvent yielded 2.0 g (92%) of an oily residue, R_f 0.74 (system 4).

20. ZThrAsp(OBu^t)GlyOH (XIV). The dipeptide (XIII) (2.0 g; 3.5 mmole) was hydrogenated in methanol over a Pd/C catalyst. After evaporation of the methanol, the residue was dissolved in 5 ml of DMFA, and 1.6 g (3.8 mmole) of ZThrOPfp was added. The reaction mixture was left for 4 h and was worked up as in experiment 19. This gave 1.3 g (82%) of an oily residue, with R_f 0.72 (system 1), 0.42 (system 4).

21. BocCys(Acm)ThrLys(Boc)ProOH (XV). The tripepetide (V) (2.0 g; 3.5 mmole) was hydrogenated in 50 ml of aqueous methanol over a Pd/C catalyst. The catalyst was filtered off, the filtrate was evaporated, the residue was dissolved in 5 ml of DMFA, and 1.8 g (3.8 mmole) of BocCys(Acm)OPfp was added. After 8 h, the reaction product was precipitated with

ether. Two recrystallizations from ether yielded 1.9 g (74%) of the tetrapeptide (XV), mp 121-124°C, R_f 0.65 (system 1), 0.62 (system 3).

22. BocCys(Acm)ThrLys(Boc)ProThrAsp(OBu^t)GlyOH (XVI). Substance (XIV) (1.3 g; 2.7 mmole) was hydrogenated in methanol over a Pd/C catalyst. The deblocked peptide was dissolved in 3 ml of DMFA, and the pentafluorophenyl ester of tetrapeptide (XV) and 2.0 g (2.7 mmole) of complex F in a mixture of 15 ml of dioxane and 5 ml of DMFA were added. The product was worked up as in experiment 3. Recrystallization from methanol led to 1.8 g (73%) of a product with mp 144-146°C, R_f 0.56 (system 1), 0.60 (system 3). Amino acid analysis: Thr 2.31 (2), Lys 0.98 (1), Pro 1.03 (1), Asp (0.92) (1), Gly 1.00 (1), Cys 0.34 (1) (decomp.).

23. BocCys(Acm)ThrLys(Boc)ProThrAsp(OBu^t)GlyAsnCys(Acm)GlyOH (XVII). Substance (XII) (0.40 g; 0.85 mmole) was deblocked as in experiment 16, and the hydrochloride so obtained was dissolved in 3 ml of DMFA and was treated with 0.11 ml (0.85 ml) of NEM and the pentafluorophenyl ester of the heptapeptide (XVI) obtained from 0.80 g (0.77 mmole) of substance (XVI) and 0.65 g (0.85 mmole) of complex F in a mixture of 9 ml of dioxane and 3 ml of DMFA. The product was worked up as in experiment 3. After two treatments with hot ethyl acetate, 0.68 g (64%) of a product was obtained with mp 160-162°c, R_f 0.43 (system 1), 0.64 (system 3).

24. Cys(Acm)ThrLysProThrAspGlyAsnCys(Acm)GlyOH (XVIIa). The deblocking of 0.55 g (0.39 mmole) of substance (XVII) by the action of 0.9 ml of 3 N HCl in dioxane in the presence of dimethyl sulfide and purification on a column of DEAE-Sephadex A-25 in an ammonium acetate buffer led to 0.35 g (67%) of the decapeptide (XVIIa), R_f 0.47 (system 2), 0.15 (system 3); E_{His} 0.72.

25. CysThrLysProThrAspGlyAsnCysGlyOH (XVIII). A solution of 0.050 g (0.042 mmole) of substance (XVIIa) in 200 ml of methanol was cooled to −40°C and was acidified with 6 N HCl to pH 5. Then a 0.1 N solution of iodine in methanol was added to the reaction mixture until it had assumed a permanent yellow coloration. After 8 h, a 0.1 N solution of sodium thiosulfate was added (until the solution had been decolorized), the methanol was evaporated off, and the product was transferred to a column of Sephadex G-25 and was eluted with 0.1 N acetic acid. The product (0.033 g; 77%) was then purified by preparative HPLC (23% of CH_3CN and 77% of 0.05% CF_3COOH). This gave 0.019 g of a substance with $[\alpha]_D^{20}$ −38.9° (c 1; 20% CH_3COOH). Amino acid analysis: Thr 2.01 (2), Lys 0.83 (1), Pro 1.09 (1), Asp 2.10 (2), Gly 2.00 (2), Cys 1.27 (2) (decomp.).

26. BocCys(Acm)GlyNHEt (XIX) was obtained from 0.6 g (5.0 mmole) of glycine ethylamide and 2.2 g (4.6 mmole) of BocCys(Acm)OPfp in 5 ml of DMFA. The product was worked up by the method of paragraph 19, which yielded 1.5 g (86%) of an oily product with R_f 0.50 (system 4).

27. BocAsnCys(Acm)GyNHEt (XX). The hydrochloride obtained from the deblocking of 1.5 g (3.9 mmole) of dipeptide (XIX) as in experiment 16 was dissolved in 3 ml of DMFA, and 0.49 ml (3.9 mmole) of NEM and 1.7 g (4.3 mmole) of BocAsnOPfp were added. The reaction mixture was left for 12 h and was then worked up as in experiment 3. Recrystallization from ethyl acetate gave 1.6 g (83%) of the tripeptide (XX), mp 116-118°C, R_f 0.62 (system 1).

28. BocCys(Acm)ThrLys(Boc)ProThrAsp(OBu^t)GlyAsnCys(Acm)GlyNHEt (XXI). The hydrochloride obtained from the deblocking of 0.44 g (0.9 mmole) of substance (XX) as in experiment 16 was dissolved in 3 ml of DMFA, and then 0.12 ml (0.9 mmole) of NEM, 0.85 g (0.81 mmole) of the peptide (XVI) and 0.68 g (0.9 mmole) of complex F were added. After 8 h, the reaction mixture was worked up as in experiment 3. Recrystallization from methanol led to 0.84 g (71%) of product with mp 151-154°C, R_f 0.49 (system 1).

29. Cys(Acm)ThrLysProThrAspGlyAsnCys(Acm)GlyNHEt (XXIa). The deblocking of 0.6 g (0.43 mmole) of substance (XXI) in a similar way to experiment 16 with purification on a column of SP Sephadex C-25 n ammonium acetate buffer (pH 5.6), led to 0.37 g (64%) of the decapeptide (XXIa), R_f 0.50 (system 2), 0.15 (system 3); E_{His} 0.74.

30. CysThrLysProThrAspGlyAsnCysGlyNHEt (XXII). A reaction by the method of paragraph 25 was conducted with 0.050 g (0.041 mmole) of substance (XXIa) and, after purification by preparative HPLC under the conditions of 25, this gave 0.022 g (49%) of substance (XXII), k' 4.86. $[\alpha]_D^{20}$ −42.4° (c 0.5; 20% CH_2COOH). Amino acid analysis: Thr 2.20 (2), Lys 0.94 (1), Pro 1.1 (1), Asp 1.93 (2), Gly 2.00 (2), Cys 1.12 (2) (decomp.).

Preparation of Conjugates. a) To a solution of 80 mg of the N-hydroxysuccinimide ester of succinylated BSA in 2 ml of DMFA were added 20 mg of a peptide and NEM to pH 8.0. The mixture was stirred for 20 h and, after the addition of a few drops of 25% ammonium solution, it was dialyzed against water and lyophilized.

b) A solution of 40 mg of a peptide in 10 ml of water and, slowly, dropwise, 0.6 ml of a 1% solution of glutaraldehyde in water were added to a solution of 60 mg of polytuftsin analogue in 10 ml of physiological solution. The mixture was stirred for 2 h and, after the addition of 20 mg of $NaBH_4$, it was dialyzed against water and lyophilized.

LITERATURE CITED

1. M. Redfern, New Sci., 121, No. 1654, 26 (1989).
2. P. Bratnagar, E. Papas, H. Blum, D. Milich, D. Nitecki, M. Karels, and G. Vyas, Proc. Natl. Aad. Sci. USA, 79, 4400-4404 (1982).
3. T. Hopp, Mol. Immunol., 21, 13-16 (1984).
4. S. Brown, C. Howard, A. Zuckerman, and M. Steward, J. Immunol. Methods, 72, 41-48 (1984).
5. G. Flouret, S. Terada, T. Kato, R. Gualtieri, and A. Lipkowski, Int. J. Peptide Protein Res., 13, No. 2, 137-141 (1979).
6. M. Bodanszky and Y. Z. Kwei, Int. J. Peptide Protein Res., 12, No. 1, 69-74 (1978).
7. S. M. Andreev, M. V. Sidorova, O. A. Rakova, D. G. Tsvetkov, and L. A. Fonina, Bioorg. Khim., 13, No. 5, 696-700 (1987).
8. Y. Trudelle, A. Brack, A. Delmas, S. Pedoussaut, and P. Rivaille, Int. J. Peptide Protein Res., 30, 54-60 (1987).

FORMATION OF PEROXIDE COMPOUNDS ON THE OXIDATION OF LIGNIN BY OXYGEN IN AN ORGANIC SOLVENT

O. V. Ignatenko, A. S. Arkhangel'skaya and É. I. Chupka

UDC 630.813.11

The results are given of a determination of the steady-state concentrations of peroxide compounds on the oxidation of lignin by oxygen in dioxane. An iodometric method of determining peroxides was used, with spectrophotometric control of the amount of iodine formed. It was established that the achievable steady-state concentration of peroxide compounds on the oxidation of lignin is 0.28-0.44 wt. % of O_{act} (oxygen pressure 1 atm, temperature 50-80°C). With a rise in the temperature, the steady-state concentration of peroxide compounds decreased. The addition of water (30 vol. %) to the organic solvent led to an increase in the rate of accumulation of peroxide compounds. It has been shown that the presence of alkaline and acidic catalysts exerts no appreciable influence on the achievable steady-state concentration and the rate of accumulation of peroxide compounds during the oxidation of lignin.

It is known that the oxidation of lignin, as a phenolic compound, is accompanied by the formation of peroxide compounds (o- and p-hydroperoxides of cyclohexadienone, quinolide perixodes, and hydrogen peroxide) [1]. Because of their high chemical activity, these peroxide compounds (PCs) are the main intermediate oxidation products participating in the further initiation and development of the chain of oxidative transformations of lignin. The instability of the hydroperoxides and the quinolide peroxides of lignin does not permit their isolation and identification as individual compounds. As a rule, iodometric methods are used to determine the stead-state concentration of PCs. The concentration of peroxides in the course of soda-oxygen digestion has been determined by such a method [2].

Bratsk Industrial Institute. All-Union Scientific Production Combine of the Pulp and Paper Industry, Leningrad.

Translated from Khimiya Prirodnykh Soedinenii, Nos. 3,4, pp. 413-417, May-August, 1992. Original article submitted October 16, 1990; revision submitted January 16, 1991.

0009-3130/92/0304-0361$12.50 © 1992 Plenum Publishing Corporation