The hydrogenolysis of 3.2 g of (XVI) gave 2.1 g (91%) of methyl 2,3,4-tri-O-methyl-α-D-galactopyranoside (XVII), $[\alpha]_D$ +125° (c 2.6; chloroform), +147° (c 3.6; methanol), n_D 1.4595. According to the literature [4]: $[\alpha]_D^{27}$ +161° (methanol), n_D^{25} 1.4626; [15]: $[\alpha]_D^{28}$ +132.2° (methanol), n_D^{25} 1.4608.

The oxidation of 1.5 g of (XVII) followed by esterification gave 1.3 g (77%) of (XVIII). Crystallization from ether yielded pure (XVIII), mp 71°C, $[\alpha]_D$ +134° (c 1.4; chloroform). According to the literature [9, 10]: mp 70.2-70.3°C, $[\alpha]_D^{26}$ +142.1° (chloroform); mp 73°C, $[\alpha]_D$ +169° (water).

SUMMARY

Unidirectional syntheses of the 2,3-, 2,4-, and 3,4-di- and 2,3,4-tri-O-methyl ethers of methyl (methyl α-D-galactopyranosid)uronate are proposed.

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CHARACTERISTICS OF A β-1,3-GLUCANASE FROM *Spisula sachalinensis* AS A GLYCOPROTEIN

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The nature of the carbohydrate-peptide bond and the composition of the carbohydrate chain in a β-1,3-glucanase from the marine mollusk *S. sachalinensis* has been investigated. According to the results of the phenol-sulfuric acid method, the neutral sugars amounted to 6.5% of the molecular weight of the enzyme. The composition of the neutral sugars (Glc : Gal : Man 5:2:1) was determined by the GLC method. It was shown that the β-1,3-glucanase molecule contains no uronic or sialic acids. The amount of amino sugars (15% with equal amounts of glucosamine and galactosamine) was established by amino acid analysis. Alkaline degradation led via the β-elimination reaction to the splitting out of 50% of the neutral sugars and showed the existence of an O-glycosidic bond in the enzyme molecule. Various actions on the carbohydrate moiety (periodate oxidation and treatment with glycosidases) caused no appreciable change in the hydrolyzing capacity of the enzyme.

In the study of the primary structure of enzymes, many of which are glycoproteins, one of the interesting aspects is the investigation of the carbohydrate moiety of the molecule;
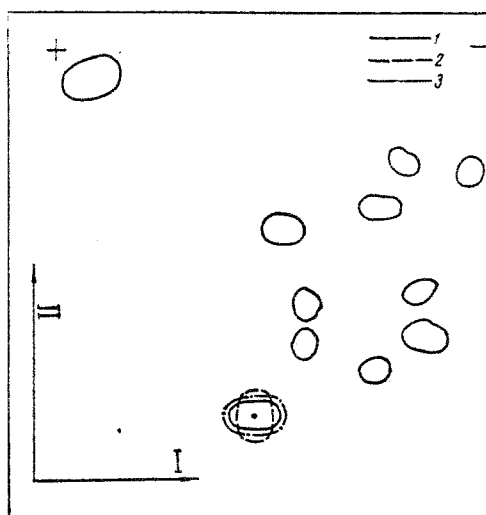
Fig. 1. Peptide map of a tryptic
hydrolysate of the reaction product
of the β-1,3-glucanase LIV with
labeled methylamine: I) electro-
phoresis, pH 5.6, V 800 V, time 50
min; II) chromatography in the butan-
1-ol—pyridine—acetic acid—water
(60:40:12:48, v/v, system). The
peptides were detected with a 0.3%
solution of ninhydrin in acetone (1);
2) label at an O-glycosidic bond; 3)
detection of neutral sugars.

the elucidation of its role in activity and the determination of the type of carbohydrate-
protein bond.

The endo-β-1,3-glucanase L IV that we are studying, which was isolated from the crys-
talline style of the marine mollusk *S. sachalinensis* in the homogeneous state [1], contains
a covalently bound carbohydrate component which is not separated on ion-exchange chromatog-
raphy or prolonged dialysis. The results of high-voltage electrophoresis of a sample of the
β-1,3-glucanase L IV hydrolysed by formic acid showed the absence of uronic acids [2]. No
sialic acids were detected by Warren's method [3]. The amino sugar content (about 15%, with
equal amounts of glucosamine and galactosamine) was determined with the aid of amino acid
analysis. The amount of neutral sugars was 6.5% according to the results of the phenol-sul-
furic acid method [4]. The qualitative composition of the neutral sugars in L IV hydrolyzed
with 2 N HCl was determined by paper chromatography. The quantitative ratio of the monosac-
charides (Glc:Gal:Man, 5:2:1) was established by the GLC method.

It is known that branched polysaccharides having α-D-mannosyl, α-D-glucopyranosyl, and
D-fructopyranosyl residues at the nonreducing ends are capable of being precipitated by con-
canavalin A (Con A). Linear α-glucans and mannans are not precipitated by Con A, although
they may be bound.

It has been established that yeast β-1,3-glucanases are glycoproteins [5, 6]. Using
homogeneous forms of three enzymes — endo-β-1,3-, exo-β-1,3-1,6-, and exo-β-1,3-glucanases,
indications of different degrees of glycosylation in them have been obtained. All the en-
zymes were close in amino acid composition and differed in the ratios of glucose and mannose
in the molecules and their capacity for being bound by Con A [7].

Under the conditions of chromatography on a column containing (Con A)-Sepharose 4B,
weak binding of the β-1,3-glucanase L IV with the Con A was observed. On immunodiffusion in
1.5% agar containing Con A [8], precipitation was observed which disappeared on washing with
0.01 M phosphate buffer, pH 7.5, containing 0.15 M NaCl. Precipitation in homogeneous solu-
tion took place at high concentrations of the enzyme (3 mg/ml) and of Con A (5 mg/ml). It
may be assumed that the observed interaction is nonspecific or weak, i.e., the carbohydrate
moiety of L IV is probably not branched or does not contain glucose and mannose residues at
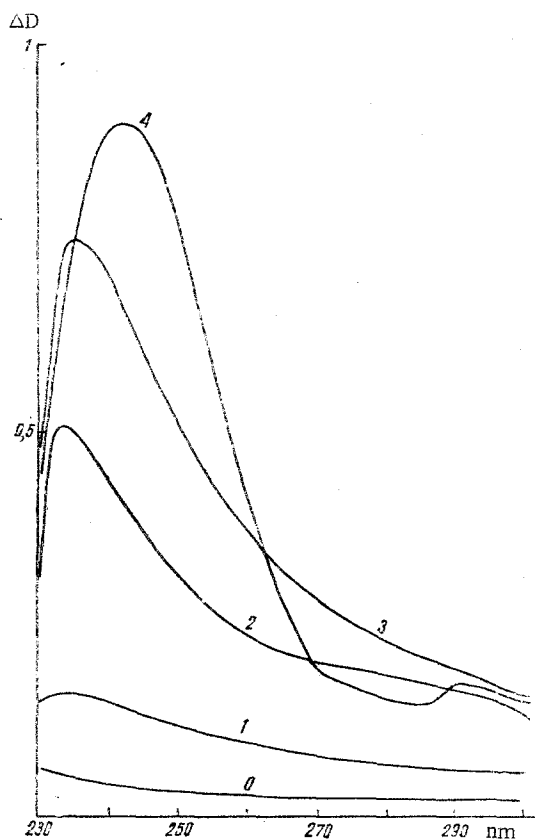the reducing ends.

ΔD

Fig. 2. Differential UV spectra of
β-1,3-glucanase L IV treated with NaOH:
0) zero line; 1) after the addition of
10 μl of 0.01 M NaOH and 25 μl of 0.05
M NaOH; 2) 40 μl of 0.05 M NaOH; 3)
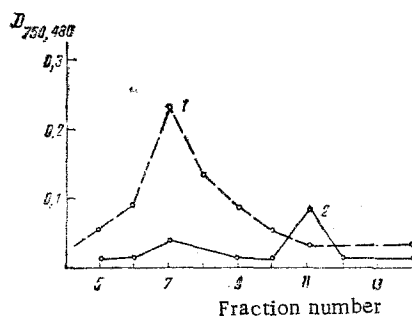60 μl of 0.05 M NaOH; 4) 10 μl of 0.1 M
NaOH.

Fig. 3. Gel filtration of a
sample of 1,3-glucanase L IV
after alkaline degradation:
1) protein; 2) amount of neu-
tral sugars.

At the present time, three main types of bonds between proteins and carbohydrates are
known: N-glycosidic bonds in which the amine group of asparagine is bound with N-acetylglu-
cosamine, O-glycosidic bonds between a sugar residue and serine, threonine, or hydroxylysine,
and O-glycosidic bonds connecting an arabinose or galactose residue with a hydroxyproline
residue [9]. Thus, for example, in the glucoamylase from *Aspergillus niger* there is an O-
glycosidic bond through the hydroxy group of a serine or threonine residue [10], and in the
saccharifying α-amylase from *Rhizopus javanicus* both O-glycosidic and N-glycosidic bonds have
been identified [11, 12].

We have performed experiments to determine the type of carbohydrate—protein bond in the molecule of the β-1,3-glucanase L IV. A reaction of the carboxymethylated β-1,3-glucanase L IV [13] with ^{14}C-labeled methylamine was performed. This reaction takes place through a stage of β-elimination with the subsequent addition of the methylamine to the double bond and is used for detecting an O-glycosidic bond in glycoproteins [14]. The labeled protein was subjected to tryptic hydrolysis and a replica was taken from the peptide map of the tryptic hydrolysate with the aid of x-ray film. The appearance of a spot at the start when the x-ray film was developed showed the presence of a O-glycosidic bond in the region of large peptides (Fig. 1). This was also confirmed by the results of the revelation of the peptide map of a tryptic hydrolysate of the native β-1,3-glucanase L IV with a reagent for sugars [15].

The decrease in absorption at 241 nm (Fig. 2) following the addition of various concentrations of a solution of caustic soda at room temperature to a solution of the enzyme indicated the alkaline degradation of the β-1,3-glucanase L IV by β-elimination [16]. The splitting out of the carbohydrate chain was confirmed by the results of the subsequent gel filtration of this sample in a column containing Sephadex G-25 (Fig. 3).

To calculate the amount of neutral sugars bound by O-glycosidic bonds in the enzyme molecule, a sample of the β-1,3-glucanase L IV was incubated in 0.05 M NaOH at 37°C. In the course of the reaction, the activity of the enzyme and the amount of sugars not split off under the reaction conditions were monitored. The determination of the neutral sugars was carried out after dialysis of an aliquot sample for 24 h. The results of the experiment showed that about 30% of the total amount of neutral sugars was split out in 30 min. With a prolonged reaction (24 h), this amount rose to 50%. This fact permits the assumption that another type of bond (for example, an N-glycosidic bond) is also present in the structure of the β-1,3-glucanase L IV. It is most likely that there is more than one point of attachment of the carbohydrate fragment in the enzyme molecule.

The question of the significance of carbohydrate components in enzymes remains open. Using the amylases of human saliva as an example, the existence of glycosylated and nonglycosylated enzymes with the same specificity has been demonstrated [17]. At the same time, in a shortened glucoamylase from *Aspergillus awamori*, which had lost part of its carbohydrate chain, the specificity had changed [18].

To determine the role of the carbohydrate component more accurately, use is frequently made of the periodate oxidation of enzymes under mild conditions. Thus, in 0.02 M sodium metaperiodate at 4°C the β-glucosidase from sweet almond lost 50% of its activity in 2 h. The total amount of monosaccharides had fallen to one half at that point [19]. In 0.01 M metaperiodate at 25°C in the dark, bacterial α-amylase lost its enzymatic activity completely in 30 min, while under the same conditions the invertase from *Candida utilis* proved to be resistant to the action of periodate [20]. The β-1,3-glucanase L IV was subjected to periodate oxidation under the conditions described [20]. In 2 h no change in enzymatic activity was determined. A fall in the activity of the β-1,3-glucanase L IV took place only when the excess of periodate was increased. However, at the same time a fall in the absorption of the enzyme solution at 280 nm, which is connected with the breakdown of the tryptophan residues, was observed. It is known that in the oxidation of glycoproteins with periodate under more severe conditions residues of the amino acids cysteine, tryptophan, methionine, and tyrosine are affected. It has been shown previously that there are no free SH groups in the β-1,3-glucanase L IV molecule [21], the tyrosine residues play no fundamental role in the activity of the enzyme [22], and the oxidation of the tryptophan leads to a fall in activity [23].

We tested the action on the β-1,3-glucanase L IV of a number of other enzymes: an N-acetylhexosaminidase from *Acmeae pallida* [24], commercial yeast α-glycosidase, and a complex of glycosidases from the terrestrial mollusk *Eulota maarkii* [25]. In the analytical variant, no influence of these enzymes on the activity of the β-1,3-glucanase L IV was detected in 24 h.

When the β-1,3-glucanase was subjected to preparative hydrolysis by α-glucosidase and by the glycosidases of *E. maarkii* the increase in the amount of reducing sugars was monitored by Nelson's method [26] and the appearance of glucose by means of glucose oxidase [27]. Monitoring by Nelson's method showed that the complex of glycosidases from *E. maarkii* hydrolyzed the carbohydrate moiety of the β-1,3-glucanase molecule in 24 h. No glucose was detected in either sample. The activity of the β-1,3-glucanase did not change. These results

confirm once more the hypothesis that glucose is not the terminal monosaccharide residue of the carbohydrate part of the β-1,3-glucanase L IV molecule.

EXPERIMENTAL

The homogeneous enzyme β-1,3-glucanase L IV from the crystalline style of the marine mollusk *Spisula sachsalinensis* [1] was used. The activity of the enzyme was determined from the increase in the amount of reducing sugars by Nelson's method [26]. Laminarin from *Laminaria cycharioides* was used as substrate. The concentration of protein was determined by Lowry's method [28] or spectrophotometrically using an absorption coefficient $E_{280}^{0.1\%} = 1$. Neutral sugars were determined by the phenol-sulfuric acid method [4], and the amount of glucose with glucose oxidase [27].

Qualitative Composition of the Neutral Sugars. A sample of the β-1,3-glucanase L IV (15-20 mg) was hydrolyzed with 2 N HCl at 100°C for 5 h. The hydrolysate, after treatment with the resins IRC-120 (H⁺ form) and DW 2 × 8 (OH⁻ form) was analyzed by descending chromatography on FN-3 paper in the pyridine—n-butanol—water (6:4:3) system. The spots were revealed with aniline phthalate and an alkaline silver solution.

The quantitative ratio of the monosaccharides in the form of the corresponding aldononitrile acetates in the same hydrolysate was determined by the GLC method, using a Pye—Unicam 104C chromatograph with a flame-ionization detector and a 10.4 × 150 cm column containing 3% of OF01 on Gas Chrom Q (I) and 3% of neopentyl glycol succinate on Chromosorb W (II).

Determination of Uronic Acids [2]. The enzyme (12 mg) was hydrolyzed with 85% formic acid at 100°C for 8 h and the reaction mixture was treated with IRC-120 resin (H⁺ form). The hydrolysate was analyzed by high-voltage electrophoresis. Electrophoresis was carried out on FN-3 paper in pyridine-acetate buffer, pH 6.5 at a voltage of 4000 V for 30 min. Galacturonic and glucuronic acids were used as markers.

Sialic acids were determined by Warren's thiobarbiturate method [3] after the acid hydrolysis of 0.5 mg of the enzyme with 0.1 N H_2SO_4 at 80°C for 40 min.

Amino sugars were determined after the acid hydrolysis of the β-1,3-glucanase L IV (2 N HCl, 105°C, 4 h) on a Biocal 3203 amino acid analyzer.

Reaction of the β-1,3-Glucanase L IV with Concanavalin A (Con A). A. A solution of the β-1,3-glucanase L IV (0.45 mg) in 0.02 M acetic acid (0.5 ml) was deposited on a column (0.8 × 13.3 cm) containing (concanavalin A)-Sepharose 4B equilibrated with 0.1 M sodium acetate buffer containing 0.5 M NaCl, 1 mM Ca^{2+}, Mg^{2+}, Mn^{2+}, pH 5, and thermostated at room temperature for 1 h. Elution was performed with the initial buffer and then with a 0.1 M solution of methyl α-D-mannoside in the buffer. The fractions were analyzed for their β-1,3-glucanase contents.

B. The immunodiffusion of the β-1,3-glucanase L IV was carried out in 1.5% agar prepared in 0.1 M sodium acetate buffer containing 0.5 M NaCl, 1 mM Ca^{2+}, Mn^{2+}, Mg^{2+}, pH 5, at a concentration of concanavalin A of 5 mg/ml and of protein of 60 μg/ml by Goldstein's method [8].

C. Precipitation in a homogeneous medium was confirmed with various dilutions of solutions of Con A (initial concentration 5 mg/ml) and of the enzyme (3 mg/ml).

β-Elimination. A. According to a method described in the literature [14], 2 mg of freeze-dried carboxymethylated β-1,3-glucanase L IV was dissolved in 0.5 ml of 8 M urea, 1 M NaOH, and a weighed amount of (^{14}C) methylamine was added to a concentration of 0.5 M. The reaction mixture was thermostated at 37°C for 1 h, after which it was neutralized with 2 N HCl. The excess of reagents was eliminated by gel filtration through a column (1.6 × 19 cm) containing Sephadex G-25 (fine). The fractions (2 ml each) corresponding to the modified protein were freeze-dried and subjected to tryptic hydrolysis.

The hydrolysate of the enzyme L IV (0.005 μmole) was deposited on a plate (20 × 20 cm) with a layer of cellulose, and electrophoresis was carried out in pyridine—acetate buffer, pH 5.6, at a voltage of 800 V for 50 min. The dried electrophoretogram was subjected to chromatography in the pyridine—n-butanol—acetic acid—water (40:60:12:48 by volume) system. The peptides were revealed with a 0.3% solution of ninhydrin in acetone. The labeled peptide was detected with the aid of an x-ray film, which was kept in contact with the plate for 12 days. A peptide map of a tryptic hydrolysate of the native β-1,3-glucanse L IV was

obtained by the method described above and was treated with a reagent for sugars [15] (see Fig. 1).

B. Increasing amounts of NaOH were added to 2 ml of a solution of the β-1,3-glucanase L IV (0.7 mg/ml) in 0.05 M sodium succinate buffer, pH 5.2, containing 0.3 M NaCl. The differential UV spectrum was recorded on a Varian Cary-219 double-beam spectrophotometer (see Fig. 2). After treatment with alkali, the sample was subjected to gel filtration through a column (2 × 17 cm) of Sephadix G-25 (fine). The fractions (1 ml) were analyzed for their protein and neutral sugar contents (Fig. 3).

C. A solution of the enzyme (5 ml, 0.95 mg/ml) in 0.05 M NaOH was kept at 37°C for a long time. During the reaction the activity of the enzyme and the amount of neutral sugars were monitored. To determine the neutral sugars, aliquot samples were subjected to dialysis for 24 h.

Periodate Oxidation. A. A solution of 1.8 g of the enzyme in 2.5 ml of 0.05 M sodium succinate buffer, pH 5.2, containing 0.3 M NaCl was subjected to periodate oxidation in 0.01 M sodium metaperiodate solution at 25°C in the dark for 5 h [20]. Aliquots were taken for determining enzymatic activity.

B. Increasing amounts of sodium metaperiodate were added to the cell of a SF-16 spectrophotometer containing 2 ml of a solution of β-1,3-glucanase L IV (0.26 mg/ml) in 0.05 M sodium succinate buffer, pH 5.2, containing 0.3 M NaCl. Simultaneously, a solution of metaperiodate was added to the control cell with 2 ml of buffer, and the activity of the enzyme and the absorption at 280 nm were monitored.

Action of Glycosidases. In the analytical variant: a mixture of 0.2 ml (1 mg/ml) of the β-1,3-glucanase L IV in 0.05 M sodium succinate buffer, pH 5.2 containing 0.3 M NaCl and 10 µl of a solution of one of the enzyme preparations — α-glycosidase, N-acetylhexosaminidase, and the combined glycosidases from *E. maarkii* — was incubated at 37°C. Samples with a volume of 10 µl were taken for determining enzymatic activity after 5, 12, and 24 h.

In the preparative variant: to 0.5 ml of a solution of the β-1,3-glucanase L IV (2 mg/ml) was added 50 µl of an enzyme preparation: 1) α-glycosidase; 2,3) the glycosidases of *E. maarkii*. After 24 h, the amounts of glucose in samples 1 and 2 were determined by the glucose oxidase method. The increase in the amount of reducing sugars in sample 3 was monitored by Nelson's method. A determination of enzymatic activity was carried out simultaneously.

SUMMARY

The β-1,3-glucanase L IV from the marine mollusk *S. sachalinensis* contains a carbohydrate component consisting of residues of neutral sugars (6.5%) and amino sugars (15%). The composition of the neutral sugars was determined by the GLC method: Glc:Gal:Man, 5:2:1. Through the β-elimination reaction, alkaline degradation led to the splitting out of 50% of the neutral sugars and showed the existence of an O-glycosidic bond in the enzyme molecule. Various actions on the actions on the carbohydrate moiety (periodate oxidation, treatment with glycosidases) did not lead to appreciable changes in the hydrolyzing capacity of the gluconase.

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SYNTHESIS OF (±)-RECIFEIOLIDE

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UDC 547.475.11+473.314

A description is given of the directed synthesis of (±)-recifeiolide (VI), starting from cyclooctene and 1-chlorobutan-3-ol. The ozonolysis of cyclooctene gave 7-formylheptanoic acid, the methyl ester of which (II) was condensed with the ylide of (3-hydroxybut-1-yl)triphenylphosphonium iodide (I) synthesized from 1-chlorobutan-3-ol. The lactonization of the 11-hydroxydodec-8(E)-enoic acid so obtained gave (VI). The IR, PMR, and mass spectra of (VI) are presented.

The total synthesis of natural macrolides has recently been attracting ever-increasing attention [1-3]. The synthesis of one of the representatives of this broad class of compounds, recifeiolide, in both the racemic and the optically active forms has been reported by a number of authors [4-6]. One of the most promising routes envisages as the key stage the synthesis of 11-hydroxydodec-8(E)-enoic acid (III) by the Wittig reaction [6].

We propose a simpler scheme based on readily available substances. 1-Chlorobutan-3-one, obtained from acetyl chloride, ethylene and $AlCl_3$ [7], was selectively reduced with $LiAlH_4$ in ether at $-78°C$ to 1-chlorobutan-3-ol. The subsequent replacement of the chlorine atom by iodine gave 1-iodobutan-3-ol. Boiling this with triphenylphosphine in benzene led to the crystalline phosphonium salt (I) with a yield of 70%.

The second fragment (II) was synthesized from the readily available butadiene dimer cyclooocta-1,5-diene. Its selective hydrogenation with hydrogen in hexane in the presence of 5% Pd/C (5% on the weight of the cyclooctadiene) at room temperature and atmospheric pressure until one equivalent of hydrogen had been absorbed permitted cyclooctene to be obtained with a yield of 95%. Ozonolysis of the cyclooctene in cyclohexane in the presence of acetic acid followed by hydrolytic cleavage of the ozonolide with acetic anhydride—sodium acetate gave 7-formylheptanoic acid, the treatment of which with diazomethane in ether gave (II) with a yield of 75-80% (after distillation).