The Journal of Biochemistry, Vol. 58, No, 6, 1965

Isolation and Identification of Enzymatic Reduction Products of D-Arabinosone by Osone Reductase

By Kihachiro Uehara, Masao Takeda and Yoshitsugu Sakata

(From the Laboratory of Biochemistry, Faculty of Pharmaceutical Sciences, Osaka University, Toyonaka)

(Received for publication, June 23, 1965)

D-Arabinosone was reduced with $NADPH_2$ by the enzyme present in the extract of baker's yeast (*1*). The crude enzyme was purified by fractionation first with ammonium sulfate and then with acetone, and separated into two kinds of enzymes by DEAE-cellulose column chromatography. They were named osone reductase [EC 1.3.1] and aldehyde reductase [EC 1.1.1], respectively, and their properties have been extensively studied (*1*, *2*).

Osone reductase catalyzed the reduction of D-arabinosone to produce both D-ribose and D-xylose as products, which were identified by paper chromatography (*1*).

However, since there was some uncertainty about the result of paper chromatography, we tried to isolate and identify these products.

Two hydrazones which were prepared from the products were identified as D-ribose hydrazone and D-xylose hydrazone.

MATERIALS

Enzyme Preparation—The enzyme was purified by ammonium sulfate fractionation, acetone fractionation and DEAE-cellulose column chromatography according to the method described previously (*1*, *2*).

D-Arabinosone—According to the modification (*3*) of Reichstein's method (*4*), D-arabinosephenylosazone prepared by Fischer's method (*5*) was decomposed with benzaldehyde. The reaction mixture was washed with ether and benzene, and then treated with active charcoal, and concentrated to a colorless syrup. The syrup was dried in vacuum, and dissolved in a small volume of absolute ethanol. After adding dried ether into this ethanol solution, the precipitate was collected in a small tube and dried in vacuum.

Isocitrate Dehydrogenase (NADP) [EC 1.1.1.42]—Acetone powder of pig heart was prepared according to Ochoa's method (*6*). The extract was fractionated with solid ammonium sulfate, and the fraction between 0.5 and 0.6 saturation was dissolved in 2 ml. of 0.04 *M* phosphate, pH 7.4. The solution was dialyzed against 2 liters of water for 3 hours at 0°C.

Hydrazines—The free bases were prepared from their hydrochlorides commercially obtained (Tokyo Kasei, Ltd.).

DL-Isocitric Acid—DL-Isocitric lactone (Sigma Chemical Company) was hydrolyzed according to the method used by Daniel *et al.* (*7*). NADP (Sigma Chemical Company), DEAE-cellulose (Brown Company) and Amberlite IR-120 ion exchange resin (Rohm & Haas Company) were commercially obtained.

D-Lyxose was prepared by the method used by Perlin and Brice (*8*). D-Arabinose (The British Drug Houses, Ltd.), D-ribose (Zellstofffabrik Waldhof) and D-xylose (E. Merck) were commercial preparations.

EXPERIMENTAL AND RESULTS

Identification of Products by Paper Chromatography—The sample solution analyzed were prepared by the following procedure.

The complete reaction mixture contained 15 μmoles of D-arabinosone, 0.35 mg. of osone reductase, 0.45 μmole of NADP, 30 μmoles of DL-isocitric acid, 10 μmoles of $MgCl_2$, 40 units of isocitrate dehydrogenase (NADP), 200 μmoles of phosphate, pH 8.0 and water in a total volume of 4.0 ml. Simultaneously the control experiments were run without NADP, D-arabinosone or osone reductase. The reaction was carried out for 15 hours at 25°C, and terminated by adding the ion exchange resin, Amberlite IR-120 (hydrogen form). The reaction mixture was concentrated to dryness under the stream of nitrogen gas below 40°C. The residue was dissolved in methanol and

the insoluble materials were removed by filtration under suction. The solution was concentrated to dryness and the final residue was analyzed by paper chromatography.

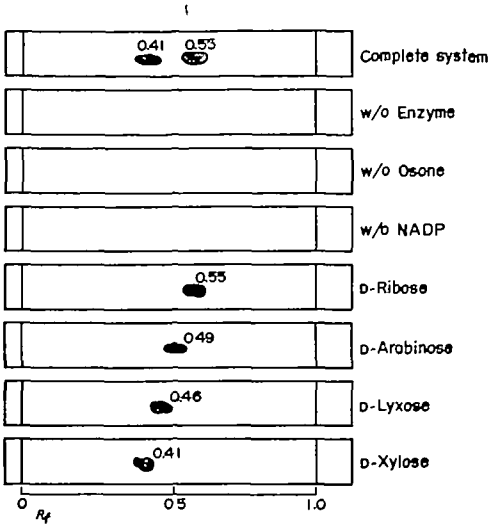


FIG. 1. Paper chromatogram of reduction products.
Solvent system: Phenol 3 and water 1 (at 25°C, ascending).
Detection: Aniline hydrogen phthalate reagent (*9*). (Spots were bright reddish).
Paper used was Toyo Roshi No. 50.

The chromatogram shown in Fig. 1, indicates that enzymatic reduction products were D-xylose and D-ribose.

Isolation of Products by Column Chromatography—Since it was necessary to further identify accurately the reduction products by other method than paper chromatography, two reduction products were isolated by column chromatography according to the method used by Khym and Zill (*10*) and were identified by preparing derivertive of each product respectively.

Dowex 1×4 (Dow Chemical Company, 100 to 200 mesh) was washed with *N* HCl, and then converted to borate form by washing with approximately 3 liters of 0.1 *M* sodium tetraborate.

The resin was equilibrated with the eluting solvent (0.02 *M* sodium tetraborate), and packed into the column of 0.85×11 cm.

The reaction mixture contained 120 μmoles of D-arabinosone, 1.0 mg. of osone reductase, 0.45 μmole of NADP, 240 μmoles of DL-isocitric acid, 10 μmoles of $MgCl_2$, 100 units of isocitrate dehydrogenase (NADP), 800 μmoles of phosphate, pH 8.0 and water in a total volume 13 ml. Reaction mixture turned to green color with the absorption maximum at 670 mμ by orcinol reaction (*11*). The reduction products were prepared as described in *Identification of Products by Paper Chromatography* and dissolved in 10 ml. of 0.01 *M* sodium tetraborate, and then applied to the Dowex column and eluted with 0.02 *M* sodium tetraborated at a rate of 1 ml. per minute. Each fraction was detected by orcinol reaction and the chromatographic pattern is shown in Fig. 2.

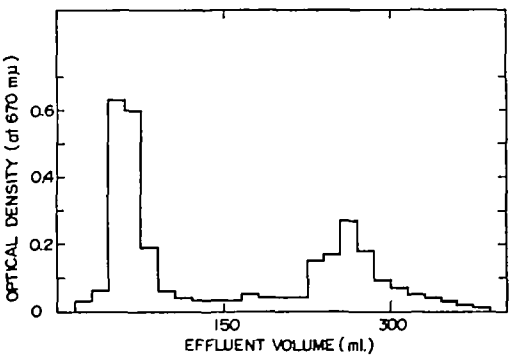


FIG. 2. The column chromatogram of enzymatic reduction products.
To 0.5 ml. of effluent was added 0.2 ml. of 0.8% orcinol solution in ethanol and 2.5 ml. of conc. hydrochloric acid containing 0.2% ferric chloride. The mixture was boiled for half an hour, and water was added to total volume 10 ml. A water blank was run simultaneously. The optical density was measured at the absorption maximum 670 mμ.

According to the determination by orcinol reaction, it was found that the fraction 3 to 7 contained about 1.6 mg. of pentose and the fraction 15 to 22 about 1.0 mg.

In order to identify the pentose sugar, each of these fractions was analyzed by paper chromatography. After removal of the cations by the ion exchange resin, Amberlite IR-120

(hydrogen form), boric acid was removed by the method used by Khym and Zill (*12*), and then each fraction was concentrated to dryness. The residues thus obtained were dissolved in 0.05 ml. of water and spotted on Toyo Roshi No. 50.

As shown in Fig. 3, two products in the reaction mixture were separated by column chromatography, and the fraction 3 to 7 had the same R_f value as D-ribose and the fraction 15 to 22 as D-xylose.

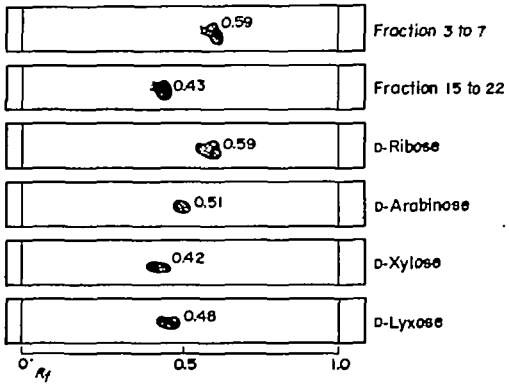


FIG. 3. Paper chromatogram of the effluent.

Solvent system : Phenol 3 and water 1 (at 25°C, ascending).

Detection : Aniline hydrogen phthalate reagent. (Spots were bright reddish).

Paper used was Toyo Roshi No. 50.

The remaining unreacted D-arabinosone, which turned to reddish brown color with the absorption maximum at 420 mμ by orcinol reaction, was eluted in the first fraction, although, as sown in Fig. 4, a small portion of it was eluted also in the pentose fractions.

In order to identify two products separated by column chromatography, 50 mg. of sugar obtained from D-ribose fraction was converted to *p*-bromophenylhydrazone and 30 mg. of sugar obtained from D-xylose fraction was converted to *p*-nitrophenylhydrazone.

Preparation of Hydrazones of Products—p-Bromophenylhydrazone of D-ribose fraction: the solution of D-ribose fraction (3 to 7) in methanol was concentrated to a small volume.

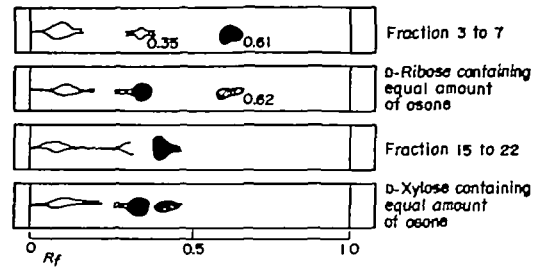


FIG. 4. Contamination of osone in solution of products.

Solvent system : Phenol 4 and water 1 (at 25°C, ascending).

Detection : $AgNO_3$—NaOH reagent (*13*).

Paper used was Toyo Roshi No. 50.

A solution of 55 mg. of *p*-bromophenylhydrazine in 10 ml. of methanol was added to it. The reaction mixture was heated at 50°C to 60°C for half an hour, and kept furthermore at room temperature overnight. After evaporation of solvent, crystals were collected and washed well with ether, and dissolved in about 10 ml. of hot water.

After cooling, small volume of ether was added and vigorously shaken. The ether layer was removed away. The water layer became colorless during this operation. After concentration of the water layer, slightly yellowish crystals were obtained. Crystals were washed with ether, and finally recrystalyzed three times from methanol. White crystals of hydrazone were obtained. Twenty five mg. of the crystals were obtained.

The crystals melted at 163°C to 164°C and at 163°C to 164°C when it was mixed with D-ribose-*p*-bromophenylhydrazone (Found; C, 41.64 : H 4.75 : N, 8.84 : Br, 24.79. $C_{11}H_{15}O_4N_2$ Br requires C, 41.39 : H, 4.72 : N, 8.77 : Br, 24.79%).

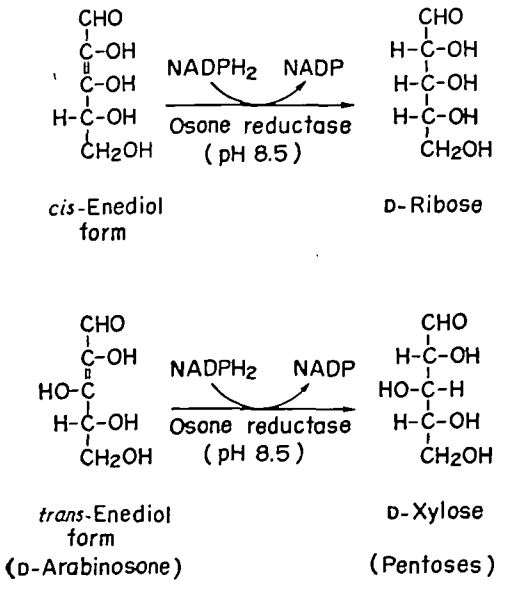
p-Nitrophenylhydrazone of D-xylose fraction : the solution of D-xylose fraction (15 to 22) in methanol was concentrated to dryness and dissolved in a small volume (approximately 1 ml.) of ethanol and 35 mg. of *p*-nitrophenylhydrazine was added to it. The reaction mixture was heated at 70°C for 10 min. The crystalization was allowed to cotinue overnight at room temperature.

Reddish yellow needle crystals were collected. Crystals were dissolved in 10 ml. of hot water, and undissolved red precipitate of osazone was filtered off. The solution was concentrated. After repeating this procedure five times, almost pure hydrazone was obtained and finally recrystalyzed from ethanol three times. Twenty three mg. of yellowish needle crystals were obtained. The crystals melted at 157°C and at 157.5°C when it was mixed with D-xylose-*p*-nitrophenylhydrazone (Found: C, 46.47: H, 5.33: N, 14.80. $C_{11}O_6H_{15}N_3$ requires C, 46.31: H, 5.30: N, 14.73%).

DISCUSSION

D-Ribose and D-xylose were identified as the reduction products of D-arabinosone by $NADPH_2$ in the presence of osone reductase, It is possible to suppose that D-arabinosone might have the structures of both *cis*- and *trans*-enediol forms, and D-ribose might be formed from the former and D-xylose from the latter.

DIAGRAM 1



Although it seems to be probable that the enzyme might not be a single enzyme, attempt to separate osone reductase into two enzymes by rechromatography in DEAE-cellulose was unsuccessful. Further studies are being carried out in order to draw final conclusion on this point.

SUMMARY

Osone reductase catalyzed the reduction of D-arabinosone to two pentoses. They were separated by column chromatography and converted into two hydrazone derivertives. One of them was identified as *p*-bromophenylhydrazone of D-ribose and the other *p*-nitrophenylhydrazone of D-xylose.

REFERENCES

(*1*) Uehara, K., and Takeda, M., *J. Biochem.*, **56**, 42 (1964)
(*2*) Uehara, K., and Takeda, M., *J. Biochem.*, **56**, 48 (1964)
(*3*) Uehara, K., Sugeno, K., Mizoguchi, T., Takeda, M., and Horiguchi, M., *J. Japan. Biochem. Soc.* (in Japanese), **34**, 290 (1962)
(*4*) Reichstein, T., Grüssner, A., and Oppenauer, R., *Helv. Chim. Acta*, **17**, 510 (1930)
(*5*) Fischer, E., *Ber. deut. chem. Ges.*, **17**, 579 (1884)
(*6*) Ochoa, S., "Methods in Enzymology", ed. by S.P. Colowick and N.O. Kaplan, Academic Press Inc. New York, Vol. **III**, p. 423 (1959)
(*7*) Daniel, H., Deutsch, and Robert, E. Phillips, "Methods in Enzymology", ed. by S.P. Colowick and N.O. Kaplan, Academic Press Inc. New York, Vol. **I**, p. 421 (1959)
(*8*) Perlin, A.S., and Brice, C., *Can. J. Chem.*, **34**, 541 (1956)
(*9*) Partridge, S.M., *Nature*, **164**, 443 (1949)
(*10*) Khym, J.X., and Zill, L.P., *J. Am. Chem. Soc.*, **73**, 2399 (1951)
(*11*) Brown, A.H., *Arch. Biochem.*, **11**, 269 (1964)
(*12*) Khym, J.X., Zill, L.P., and Chiae, G.M., *J. Am. Chem. Soc.*, **75**, 1339 (1953)
(*13*) Smith, I., "Chromatographic Techniques", ed. by I. Smith, William Heinemann Medical Books. Ltd., London, p. 164 (1958)