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STAPHYLOCOCCUS CARNOSUS ALDOLASE AS CATALYST FOR ENZYMATIC ALDOL REACTIONS

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SUMMARY: The substrate specificity of S.carnosus aldolase is reported. 5,6-Dideoxyhexulose was synthesized in preperative scale from dihydroxyacetone phosphate and propionaldehyde. The product was isolated and characterized by NMR.

Aldolases are the most useful enzymes for the synthesis of monosaccharides and related compounds.[1,2,3] The most thoroughly studied class I aldolase is the fructose 1,6-bisphosphate aldolase from rabbit muscle (RAMA) (E.C.4.1.2.13). This enzyme is quite specific for dihydroxyacetone phosphate (DHAP) as one substrate, but will accept a wide variety of aldehydes for condensation.[4] The enzyme from rabbit muscle, however, suffers from (1) instability during synthesis of sugar phosphates and (2) loss of activity in the presence of many reactor materials.[5] We described recently a very stable class I fructose 1,6-bisphosphate aldolase from Staphylococcus carnosus.[6] In this paper we report the substrate range and the stability of the enzyme during synthesis of sugar phosphates in comparism to RAMA.

Purification: S.carnosus was cultivated as previously described.[6] A new purification procedure for S.carnosus aldolase was developed, applicable in large scale. Extraction: An aqueous two phase system[7] was established with final concentration of 20% cellhomogenate, 27% PEG 400 and 7% KH_2PO_4/ K_2PO_4 pH 7.5. The clear top phase of the two phase system was collected containing more than 95% of the original aldolase activity.
Q-Sepharose chromatography I: The top phase was diluted 4-fold in 20 mM Tris-HCl buffer pH 7.5 and applied on a Q-Sepharose column (3x7 cm), previously equilibrated with 20 mM Tris-HCl buffer pH 7.5 containing 0.1% mercaptoethanol (standard buffer). Protein was eluted by a step gradient

of the same buffer containing 0.25 M NaCl.
Q-Sepharose chromatography II: The peak fractions were pooled, diluted 2-fold with standard buffer and applied on the same Q-Sepharose column, equilibrated with standard buffer containing 0.15 M NaCl. A linear gradient of 0.15- 0.25 M NaCl was used for elution. Aldolase eluted at 0.2 M NaCl concentration. The final yield was 70%, the specific activity 13 U/mg. The enzyme was lyophilized and stored at -20° C.

Table 1: Substrates of S.carnosus aldolase

substrates	substrates
formaldehyde	butyraldehyde
acetaldehyde	2-methylbutyraldehyde
chloroacetaldehyde	3-methylbutyraldehyde
glycolaldehyde	3-ketobutyraldehyde
glyoxylic acid	valeraldehyde
propionaldehyde	isovaleraldehyde
D,L-glyceraldehyde	trimethylacetaldehyde
D-glyceraldehyde	phenylacetaldehyde
L-glyceraldehyde	malondialdehyde
3-methylmercapto-propionaldehyde	phthaldialdehyde
methylglyoxal	2-pyridincarboxaldehyde
glyceraldehyde 3-phosphate	4-pyridincarboxaldehyde

Substrate specificity. DHAP was synthesized by the method of Effenberger and Straub (1987).[8] An aqueous solution of 20 mM DHAP, 200 mM of various aldehydes and 1-4 U/ml purified S.carnosus aldolase was incubated for 5-7 hours. The resulting sugar phosphates were detected after thin layer chromatography with acid molybdate reagent (Polygram CEL 300 20x20 cm from Macherey & Nagel, Düren; developed with 51.25 ml butanol: propanol: aceton: water 4:2:2:2.5 + 3.75 ml formic acid and 12 g trichloroacetic acid). 38 Aldehydes were tested as aldolase substrates. Table 1 summarizes those, which are converted by the S.carnosus aldolase. In general aliphatic aldehydes are good substrates but aromatic do not react. Furthermore we found increasing activation of the carbonyl group by electron-withdrawing substituents, eg: chloroacetaldehyde is more reactive than acetaldehyde. Such an effect is also well known for the substrate

specificity of RAMA.[4] The following aldehydes were not converted by the enzyme under the conditons specified above: benzaldehyde, o-toluylaldehyde m-toluylaldehyde, p-toluylaldehyde, o-hydroxybenzaldehyde, m-hydroxybenzaldehyde, p-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, m-phenoxybenzaldehyde, 3-pyridinecarboxaldehyde, 4-fluorobenzaldehyde, 4-chlorobenzaldehyde, 4-bromobenzaldehyde, 4-cyanobenzaldehyde. Taken together the results demonstrate, that the bacterial aldolase similar to RAMA has a rather large substrate range with regard to the aldehyde component.

Stability of S.carnosus aldolase during synthesis. Fig.1 shows that S.carnosus aldolase is considerably more stable under reaction conditions than RAMA. The inactivation rate for S.carnosus aldolase is 0.8%/d, while that for rabbit aldolase is nearly 60%/d.

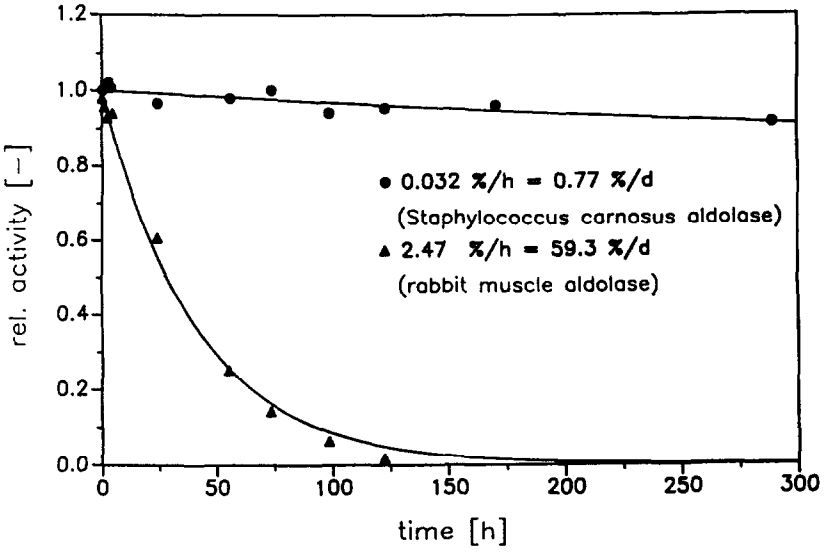

Fig.1: Stability of S.carnosus- and rabbit muscle aldolase in synthesis. Conditions: 20 mM DHAP and 75 mM glyoxylic acid were incubated at pH 6 at 25° C with a) 2.9 U/ml S.carnosus aldolase (25 U/mg) and b) 3.4 U/ml RAMA(9 U/mg).

Substrate range and stability of the S.carnosus aldolase make the bacterial enzyme the catalyst of choice for large scale synthesis of glycosidase inhibitors[3] and similar compounds with multiple chiral centers.[9] The enzyme can be prepared in large amounts by the method described above.

Aldolase catalyzed reaction of DHAP and propionaldehyde to 5,6-dideoxy-hexulose 1-phosphate. An aqueous solution (100 ml) of 50 mM DHAP, 500 mM propionaldehyde and 40 U of purified aldolase in 60 mM Tris-HCl buffer pH 6.8 was incubated at 25° C for 5.5 hours. Excess propionaldehyde was removed passing argon through the solution, DOWEX WX 8 H^+ was added to adjust the pH to 5. The protein was precipitated with 100 ml ethanol and the solvent was concentrated in vacuo to a final volume of 20 ml. After addition of 200 ml n-butanol, 1 g cyclohexylammonium sulphate and 2 ml cyclohexylamine, the solution was allowed to stand over night at -20° C. After filtration of insoluble sulphates, 200 ml of diethyl ether were added at 6° C, to cristallize the white product, which was centrifuged, washed with ether and dried in vacuo. The final yield was 1.3 g (65%).

Synthesis of 5,6-dideoxyhexulose. 500 mg of 5,6-dideoxyhexulose 1-phosphate cyclohexylammonium salt were dissolved in 30 ml distilled water and pH was adjusted to 5 with DOWEX WX 8 H^+. 50 U of acid phosphatase were added under argon atmosphere. After 5 hours the protein was denaturated by heating at 75° C. The solution was concentrated to a volume of 20 ml and extracted twice with 50 ml ethyl acetate. The aqueous layer was evaporated under reduced pressure nearly to dryness and then extracted three times with 50 ml portions of boiling acetone. The combined organic layers were dried over sodium sulphate and concentrated in vacuo yielding 160 mg of a yellow oil (86%). The "yellow oil" was acetylated by standard procedure (50 ml abs. pyridine, 25 ml acetic anhydride) and identified as 1,3,4-tri-O-acetyl-5,6-dideoxyhexulose by ^{1}H- and ^{13}C-NMR spectroscopy.

^{1}H-NMR (360 MHz, $CDCl_3$):
δ=4.70 (d,H-1a), 4.83 (d,H-1b), 5.29 (d,H-3), 5.22 (ddd,H-4), 1.62 (ddd, 2-H,CH_2), 0.87 (dd,3H,CH_3).
$J_{1a,1b}$=17.3, $J_{3,4}$=3.1, $J_{4,5}$=21.5, $J_{5,6}$=7.4 Hz.

^{13}C-NMR (360 MHz, $CDCl_3$):
δ=57.4 (C-1), 198.1 (C-2), 13.8 (C-3), 67.5 (C-4), 64.0 (C-5), 0.0 (C-6), 11.5, 12.4 (CH_3-CO), 170.2, 170.8, 171.0 (CH_3-CO).

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REFERENCES

1) C.H. Wong, G.M. Whitesides, J.Org.Chem., 48, 3199 (1983).
2) J.R. Durrwachter, H.M. Sweers, K. Nozaki, C.H. Wong, Tetrahedron Lett., 27, 1261 (1986).
3) T. Ziegler, A. Straub, F. Effenberger, Angew.Chem., 100, 737 (1988).
4) M.D. Bednarski, E.S. Simon, N. Bischofsberger, W.D. Fessner, M.J. Kim, W. Lees, T. Saito, H. Waldmann, G.M. Whitesides, J.Am.Chem. Soc., 111, 627 (1989).
5) B. Bossow-Berke, Dissertation, Universität Bonn (1989).
6) H.P. Brockamp, M.R. Kula, Appl.Microbiol.Biotechnol., in press.
7) M.R. Kula, K.H. Kroner, H.Hustedt, Adv.Biochem.Eng., 24, 73 (1982).
8) F. Effenberger, A. Straub, Tetrahedron Lett., 28, 1641 (1987).
9) M. Schultz, H. Waldmann, W. Vogt, H. Kunz, Tetrahedron Lett., 31, 867 (1990).

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