Preparation and Purification of Fructose-1,6-diphosphate

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Some investigations have been made concerning the accumulation of fructose-1,6-diphosphate (FDP) by yeasts. The concentration of glucose-6-phosphate and fructose-6-phosphate during fermentation has been determined. Optimum conditions for the precipitation of FDP have been determined and some of the impurities of Ca_2FDP have been analyzed. A new simple purification method is introduced.

Fructose-1,6-diphosphate (FDP) is a well-known intermediate of sugar metabolism. It is accumulated in a fermentation mixture of yeast, sugar, inorganic phosphate and toluene.^{1,2} FDP is recovered from the solution usually as a Ca- or Ba-salt. Complete precipitation occurs when ethanol is added to the solution.³ Crude salts contain many impurities derived from yeast and starting materials. FDP has been purified by acid and charcoal treatment^{3,4} or by ion exchange.⁵ The purity achieved by these methods has been 70–80 %. To obtain pure FDP it is usually converted to salts with organic bases.^{6,7} The main aim of this work was to find optimum conditions for the precipitation of FDP and to develop a simple purification method.

MATERIAL AND METHODS

FDP was prepared by fermentation using fresh brewer's yeast, sucrose, NaH_2PO_4 and toluene. Optimum conditions for FDP accumulation were determined earlier⁸ and were as follows: pH 6.6, temperature 30 °C, the amount of yeast (25 % dry matter) 500 g in 1000 g of reaction mixture, sucrose concentration 7–8 % (w/w), and concentration of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 5–6 % (w/w). About 45 ml toluene in 1000 g of reaction mixture were needed for maximum FDP accumulation.

After fermentation the proteins were precipitated with 100 ml of 80 % trichloroacetic acid per 1000 g of reaction mixture. The mixture was

centrifuged, neutralized and filtered. The concentration of FDP in the clear solution was 50–55 g/l. FDP was precipitated as a Ca-salt using 50 % (w/w) CaCl_2 solution. Final precipitation occurred when ethanol was added to the mixture. Ca_2FDP was filtered off from the mixture and washed with 70 % ethanol and subsequently with 94 % ethanol. The product was freeze-dried.

FDP was determined enzymatically according to the method of Bergmeyer.⁹ Commercial $\text{Na}_3\text{FDP} \cdot 8\text{H}_2\text{O}$ (Boehringer Mannheim, GmbH) was used as standard. Inorganic and total phosphorus was determined by Allen's¹⁰ method. Nitrogen content of the precipitated Ca_2FDP was determined by the Kjeldahl method with an ammonia specific electrode. A thermoanalyzer was used to determine the water content of Ca_2FDP . Fermentable sugars¹¹ and sugar phosphates¹² were separated by ion exchange and determined by an autoanalyzer¹³ using the anthrone method. The flow sheet of the reverse osmosis system used in the purification tests is presented in Fig. 1. Cellulose acetate membranes prepared according to van Oss¹⁴ were used. The heat treatment of the membranes was accomplished by incubating the membranes in hot water for 4 min.

Fig. 1. The reverse osmosis system. A compressed nitrogen, B stirred tank, C magnetic stirrer, D heat exchanger, E pump, F manometer, G reverse osmosis unit, H concentrate and J permeate.

RESULTS

Only yeast that had been used in beer fermentation was able to accumulate FDP. Fresh unused brewer's yeast, fresh baker's yeast, *Rhodotorula glutinis*, and *Candida utilis* did not cause accumulation of FDP. The ability of the yeast to accumulate FDP depended on the length of time it had been used in beer fermentation. During ten days' fermentation the yeast's ability to produce FDP increased and reached its maximum level during the last few days. The conversion of sucrose to FDP was about 15 % when using yeast from the suspension in the fermentation tank and about 40 % with the yeast settled at the bottom of the tank. The yeast used in this work had passed 3–5 beer fermentations. When FDP was produced the end point of the fermentation was determined by measuring the concentration of inorganic phosphate in the reaction mixture (Fig. 2). The FDP-concentration of the mixture reached maximum with the lowest concentration of inorganic phosphate.

Glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P) were also found in the fermentation mixture. Shortly before the end point of the fermentation their concentrations were quite high. During fermentation samples were taken from the reaction mixture as shown in Fig. 2 (black dots) for the determination of monophosphates (Fig. 3).

The precipitated Ca_2FDP was purest in the experiments with the highest initial sugar concentration (Table 1) and with an amount of

Fig. 2. The concentration of FDP (●) and inorganic phosphate (calculated as $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) (○) in the reaction mixture during fermentation.

Fig. 3. The amount of G-6-P and F-6-P in the mixture during fermentation. The pictures from top to bottom refer to the sampling times 1, 2, 3, and 4 given in Fig. 2, in this order. Dilutions were 1:10 (—) and 1:1 (---).

Table 1. Dependence of purity of the precipitated Ca_2FDP on the initial sugar concentration.

Initial sugar conc. g/l	Conc. of FDP g/l	Purity of Ca_2FDP %	P_{inorg} %	N %
50	29	69	2.2	0.47
81	52	79	0.9	0.34

inorganic phosphate somewhat below the optimum. Inorganic phosphate precipitated as an impurity when FDP was recovered. Therefore it is advisable to stop the reaction when the concentration of inorganic phosphate is lowest.

FDP was recovered as a Ca-salt. Final precipitation occurred when ethanol was added. When the volume of ethanol used was equal to the volume of fermentation solution most of the

Table 2. Precipitation of FDP from 50 ml of fermentation solution with 5 ml of 50 % (w/w) CaCl_2 and ethanol. The concentration of FDP was 42.5 g/l.

Ethanol ml	Yield g	Purity of Ca_2FDP %	P_{inorg} %	N %
—	2.2	65	1.19	0.41
40	4.9	58	0.41	0.45
50	4.8	63	0.48	0.46
70	4.9	64	0.60	0.44
100	4.4	64	0.78	0.47

Ca_2FDP precipitated (Table 2). Ca_2FDP precipitated instantly and could be filtered off immediately. The purity of Ca_2FDP , precipitated as mentioned above, was about 70 %. The water content of the product was 5–7 %, $\text{Ca}_3(\text{PO}_4)_2$ content 1–2 %, the amount of nitrogenous compounds (6.25N) 2–5 %, and the amount of monophosphates less than 1 %. The rest of the impurities were organic compounds.

The purification tests were aimed at finding a simple, preliminary method to purify the fermentation solution. Charcoal treatment, ion exchange, and ultrafiltration were tested. Best results were obtained when the solution was concentrated by reverse osmosis (Fig. 1). Because of the technical limitations of the system used the solutions were diluted with water before concentration. The results obtained with membranes heated at different temperatures are shown in Fig. 4.

The retention of FDP was at least 99 % with all these membranes. By diluting the fermentation solution before concentration a purer product was obtained. The purity was increased by washing the concentrate with water. Table 3 shows the effect of dilution of the fermentation solution and of washing of the concentrate on the purity of the precipitated Ca_2FDP .

By the above method 13–15 % of the impurities were removed. The purification did not affect inorganic phosphate or nitrogen content of Ca_2FDP . Most of the nitrogenous compounds and the colour of the product could be removed by treating the concentrate with charcoal. After this, there were still at least 5 % of unknown impurities in the product. Some of these could be removed by ion exchange. The composition of the purified product was as follows:

Fig. 4. Concentration of fermentation solution by reverse osmosis. Initial FDP-concentration and heat treatment of the membranes were 23 g/l, 66 °C (○); 18 g/l, 70 °C (●), and 23 g/l, 77 °C (●). The volume and FDP-concentration of the permeate is presented as a function of time.

Ca_2FDP	83–85 %
H_2O	6–7 %
$\text{Ca}_3(\text{PO}_4)_2$	1–2 %
6.25N	0.2–0.5 %
Monophosphates	1 %
	92–95 %

DISCUSSION

Obviously the membrane of the yeast has to be modified or partly destroyed to make the accumulation of FDP possible. The permeation characteristics of the membrane are changed by drying¹⁵ or by plasmolytic agents, e.g. toluene. Addition of toluene is not always necessary when using dry yeast.³ Some fresh baker's yeasts fail to cause any accumulation of FDP whilst others produce considerable amounts of this compound in the same procedure.¹⁶ The membrane of the yeasts causing accumulation of FDP may be more sensitive to toluene or

Table 3. Purification of the fermentation solution with a cellulose acetate membrane (heat treatment 72 °C). The pressure was 2.9 MPa (30 kp/cm²) and the temperature 22 °C. Initial volume was 700 ml and final volume of the concentrate 200 ml.

Initial conc. g/l	Washing ml H ₂ O	Purity of Ca ₂ FDP %	P _{inorg} %	N %
50 ^a	—	71	0.24	0.46
25	—	74	0.24	0.49
12	—	78	0.22	0.45
11	2 × 500	84	0.40	0.59

^a Reference sample, not concentrated.

other plasmolytic agents. According to our experience even results obtained with a single yeast strain depend very much on the history of the yeast, *e.g.* its previous use in the brewing process. Many years ago Meyerhof¹⁷ proved that the slow fermentation of FDP was caused by the destruction of the sensitive and structurally bound adenylypyrophosphatase, since adenosine triphosphate can be split only by means of this enzyme in the absence of a phosphate acceptor. However, this does not explain the considerable stimulation of the accumulation of FDP by an addition of a phosphate acceptor, *e.g.* adenosine monophosphate.⁵ A similar stimulation has been found to occur with several purine compounds. Thus the detailed reasons for an efficient accumulation of FDP still seem to be somewhat unclear.

When optimum amounts of starting materials are used in the reaction mixture and the reaction is stopped at the right moment a purer product is obtained (75 %) than with earlier preparation methods. The use of maximum sugar concentration and somewhat less than an optimum amount of inorganic phosphate is important. The reaction should be stopped at the highest FDP concentration, since the amount of inorganic phosphate is lowest at this point and thus only a small amount of Ca₃(PO₄)₂ precipitates as an impurity with FDP. At the same time the concentration of monophosphates is also low. On the other hand, a slightly shorter reaction time might also offer a new possibility for preparation of G-6-P and F-6-P.

The purity of the product increases consider-

ably when the fermentation solution is diluted and then concentrated by reverse osmosis before the precipitation of FDP. Washing of the concentrate with water increases the purity. Most of the unknown impurities are removed by this method. However, in some cases a considerable amount of FDP (0–50 %) disappeared during the concentration. The reason for this is not known; no FDP was found in the permeate.

Most of the unknown impurities of the purified Ca₂FDP were organic compounds. In X-ray diffraction analysis only phosphorus and calcium could be detected. Only traces of other elements (<1 %) were present.

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