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Effect of Ethanol on Formation of Cyclodextrin from Soluble Starch by *Bacillus macerans* Cyclodextrin Glucanotransferase

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Formation of cyclodextrin (CD) from soluble starch by *Bacillus macerans* CGTase (cyclodextrin glucanotransferase) was studied experimentally in both systems with and without the addition of ethanol. The yields of α-, β- and γ-CD after long time reaction were almost independent of initial concentration of starch (5–50 g/l) in both the systems. The α-CD yield in the presence of ethanol was increased by 1.8–1.9 times of that in the absence of ethanol, while the β-CD yield was decreased by the amount almost comparable to the increase in α-CD yield. Initial rates of CD formation were decreased with increasing ethanol concentration, which was remarkable especially for α-CD.

Die Wirkung von Ethanol auf die Bildung von Cyclodextrin aus löslicher Stärke durch *Bacillus macerans*-cyclodextrin-glucanotransferase. Die Bildung von Cyclodextrin (CD) durch Bacillus macerans-CGTase (Cyclodextrin-glucotransferase) wurde experimentell in zwei Systemen, d. h. mit und ohne Zusatz von Ethanol, untersucht. Die Ausbeuten an α-, β- und γ-CD nach Langzeitreaktion waren in beiden Systemen fast unabhängig von der anfänglichen Stärkekonzentration (5–50 g/l). Die Ausbeute an α-CD erhöhte sich bei Gegenwart von Ethanol auf das 1,8–1,9fache im Vergleich zur Abwesenheit von Ethanol, während die β-CD-Ausbeute sich um fast den gleichen Betrag verringerte. Die Anfangsgeschwindigkeiten der CD-Bildung verringerten sich mit zunehmender Ethanolkonzentration, was insbesondere bei α-CD zu beobachten war.

1 Introduction

It is well known that formation of cyclodextrin (CD) from starch by CGTase (cyclodextrin glucanotransferase) is strongly influenced by the addition of organic compounds such as surfactants [1], alcohols [2, 3], ethers, esters and ketones [3]. This is directly characterized by changes in yields of α-, β-, and γ-CD. *Kobayashi* et al. [1] have studied the effect of various kinds of surfactants on the CD yields, and found that these substances possess the ability to increase preferentially the yield

of a specific CD. They have thus concluded that such surfactants readily twist linear chains of starch molecules into helical structures with 6 or 7 glucosidic residues per one cycle. As for other organic compounds, however, a sufficient understanding has not yet been made.

In Japan, industrial production of CDs has been carried out with more than 600 t/a, and most of the products have been employed in the food field. However, the use of CDs as a food additive has been confined to only those manufactured either without the addition of organic compounds or with the addition of ethanol. In this paper, therefore, we examine experimentally the effect of ethanol on enzymic formation of CDs.

2 Materials and Method

CGTase from *Bacillus macerans* was donated by Amano Pharmaceutical Co. Ltd., Nagoya, Japan. The enzyme activity was 202 units/ml; one unit means the amount of the enzyme that produces one μmol of α-CD per min from 50 g/l of soluble starch at 50°C and pH 6.0. Soluble starch from white potato (biochemical grade) was purchased form Wako Jun-yaku Co. Ltd. (Osaka, Japan). All the experiments were carried out at *50°C* and *pH* 6.0 by stirring magnetically a reaction mixture in an Erlenmeyer flask with three baffles.

A small amount of the reaction mixture was withdrawn at appropriate intervals and kept for 10 min in boiling water to inactivate the enzyme. The liquid sample was mixed with an equal volume of acetonitrile to precipitate high-molecular weight components, and was filtered through a membrane with a pore size of 0.45 μm. The filtrate was used to determine the concentrations of α-, β-, and γ-CD and maltodextrins such as glucose, maltose and maltotriose using a HPLC, equipped with an Amide 80 separation column (Tosoh Co. Ltd.; Japan) kept at 50°C, and a refractometer. Acetonitrile-water (70 : 30) solution was used as a mobile phase at a flow rate of 1 ml/min.

3 Results

3.1 Effect of starch concentration

Starch molecules at a higher concentration usually tend to retrograde at a higher rate. The retrogradation may also be accelerated in the presence of organic solvents such as ethanol. Although CGTase is capable of acting even on starch molecules that have retrograded, it is very important to know to what extent the enzymatic action is inhibited by the addition of ethanol. To confirm this, the concentrations of respective CDs, which have been produced with the initial concentrations of starch ranging from 5 to 50 g/l, were compared between the cases with and without the addition of ethanol. As an example, time courses of the α-CD concentration are shown in Figs. 1 a and b. In all the runs without ethanol, the α-CD concentrations reached a maximum and then decreased at a relatively high rate. In the runs with ethanol, however, the degradation of α-CD was obviously restrained.

Figs. 2 a and b show the relationships between the concentrations of α-, β-, and γ-CD, and a sum of these CDs (T-CD) after 24 and 75 h and the initial concentration of starch in the absence and presence of ethanol, respectively. The α-CD concentrations both after 24 and 75 h were higher in the presence than in the absence of ethanol, but with β-CD and γ-CD the relation was quite inverted. It should be noted here that, in the absence of ethanol, the α-CD concentration after 75 h showed almost the same level as the β-CD concentration. There was an almost linear relation between all the concentrations of CDs and the initial concentration of starch. This fact suggests that, as for the

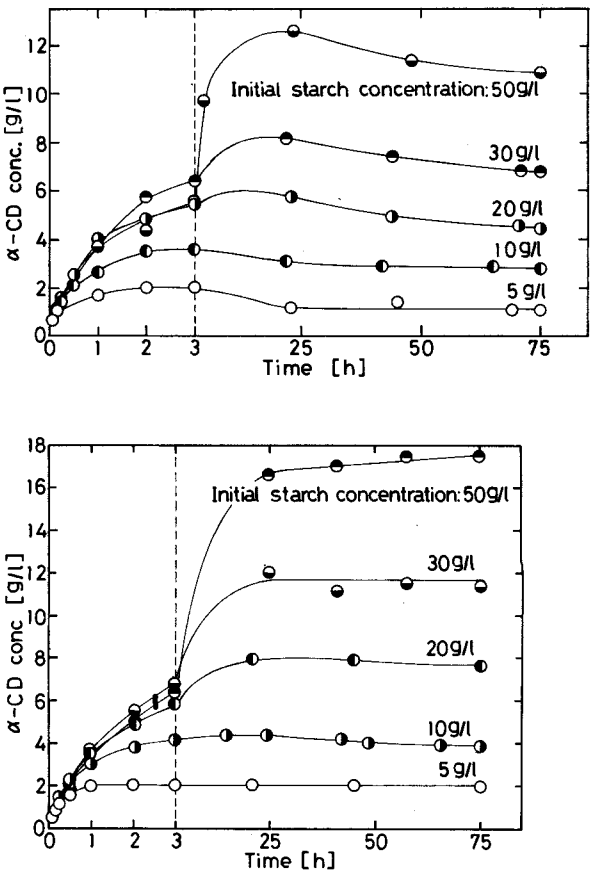


Fig. 1. Time courses of α-CD concentration (a) in the absence of ethanol and (b) in the presence of ethanol (4% v/v).
Enzyme concentration; 0.36 ml/l.

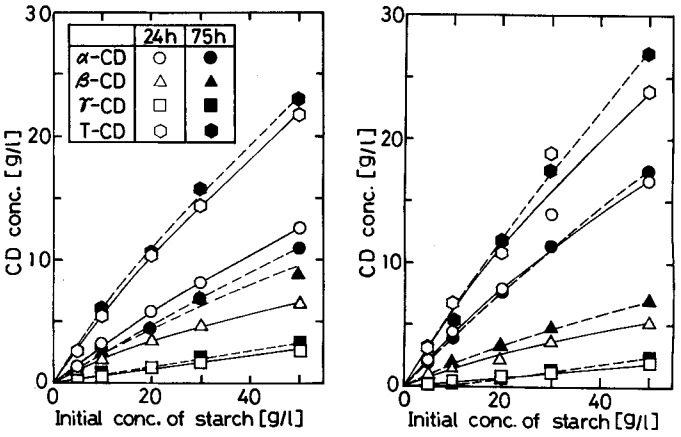


Fig. 2. Relationship between CD concentrations and initial concentration of starch (a) in the absence of ethanol and (b) in the presence of ethanol (4% v/v).
Enzyme concentration; 0.36 ml/l.

CD yields after long time reaction, the action of CGTase was substantially free from the influence of the retrogradation of starch molecules by ethanol in the range of starch concentration investigated.

3.2 Effect of enzyme concentration

The effect of enzyme concentration ranging from 0.18 to 12 ml/l on time-transient behaviors of the CD formation was investigated at a fixed initial starch concentration of 10 g/l. Typical results are illustrated in Figs. 3–6. In the absence of ethanol, the α-CD concentration was increased to a maximum and then decreased with time in all the ranges of enzyme concentration; both of these increase and decrease rates became higher as the enzyme concentration was increased.

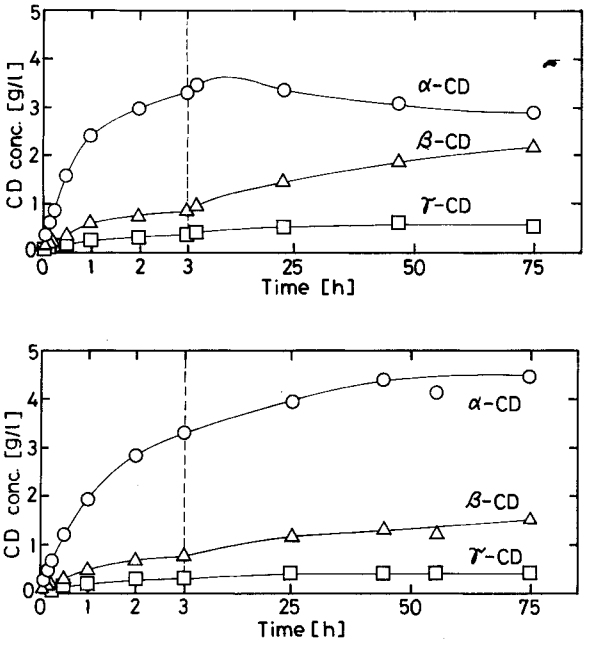


Fig. 3. Time courses of α-, β- and γ-CD concentrations (a) in the absence of ethanol and (b) in the presence of ethanol (4% v/v). Enzyme concentration; 0.18 ml/l, intial concentration of starch; 10 g/l.

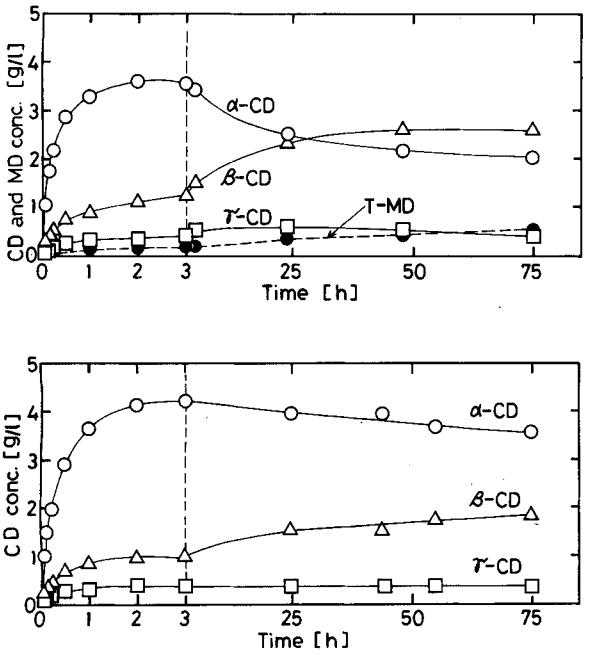


Fig. 4. Time courses of α-, β-, and γ-CD and T-MD concentrations (a) in the absence of ethanol and (b) in the presence of ethanol (4% v/v). Enzyme concentration; 0.72 ml/l, initial concentration of starch; 10 g/l.

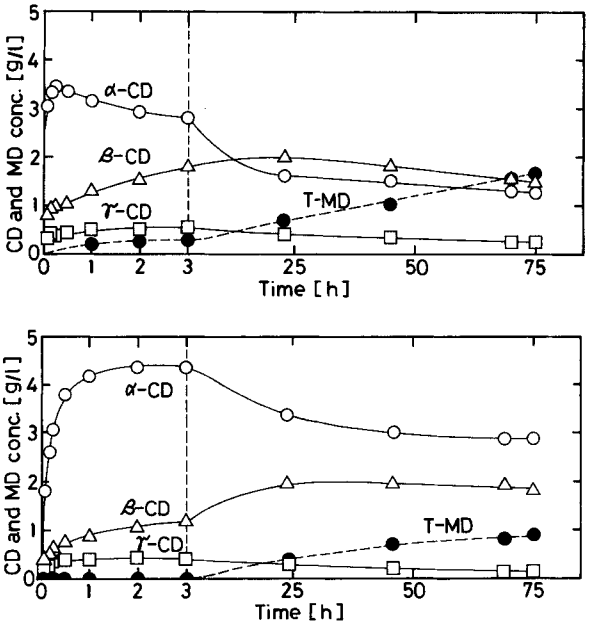


Fig. 5. Time courses of α-, β-, and γ-CD and T-MD concentrations (a) in the absence of ethanol and (b) in the presence of ethanol (4% v/v). Enzyme concentration; 2 ml/l, initial concentration of starch; 10 g/l.

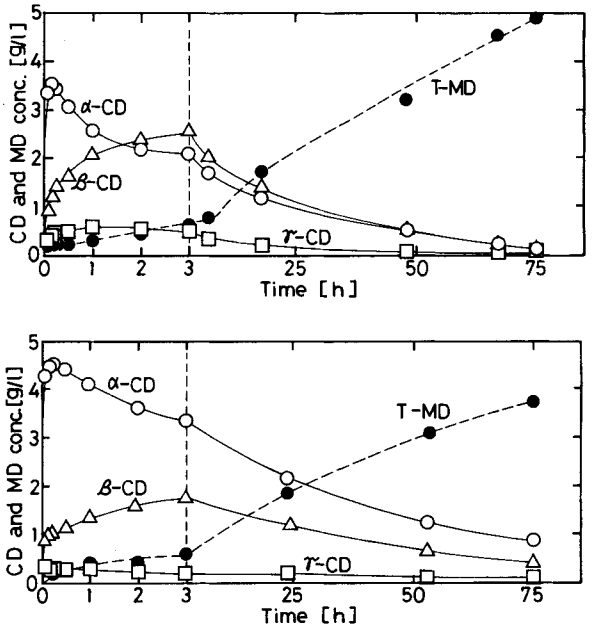


Fig. 6. Time courses of α-, β-, and γ-CD and T-MD concentrations (a) in the absence of ethanol and (b) in the presence of ethanol (4% v/v). Enzyme concentration; 12 ml/l, initial concentration of starch; 10 g/l.

Whereas, the α-CD concentration in the presence of ethanol was always increased to the value above 4 g/l, and then declined more slowly except at the enzyme concentration of 0.18 ml/l. The β-CD concentration in the absence of ethanol was increased monotonically to an almost equal level to the α-CD concentration after 75 h in the region of low enzyme concentration; but, at a sufficiently high enzyme concentration, it was decreased rapidly after being increased until about 3 h. When ethanol was added, the β-CD production was of a lower concentration. The degradation of each CD, when the enzyme concentration was 12 ml/l, was almost completed after 75 h,

regardless of the addition of ethanol. As a result of the CD dissipation, various kinds of maltodextrins were produced in large quantities. The majority of these products was glucose, maltose and maltotriose. Figs. 4a, 5 and 6 also include time courses of the maltodextrin formation in terms of the total concentration of the three products (T-MD). The increase in T-MD concentration can be well understood by the changes in α-CD and β-CD concentrations. It is clear that the maltodextrin formation was suppressed in the presence of ethanol.

3.3 Effect of ethanol concentration

The effect of ethanol concentration on time courses of the CD concentrations was investigated under the constant enzyme and initial starch concentrations. As an example, the result for the α-CD is represented in Fig. 7. It was intuitively obvious that the

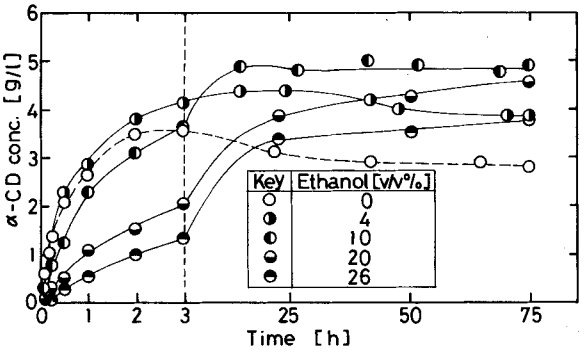


Fig. 7. Effect of ethanol concentration on time-transient behaviors of α-CD concentration.
Enzyme concentration; 0.36 ml/l, initial concentration of starch; 10 g/l.

initial reaction kinetics as well as the time course kinetics of the CD formation were considerably affected by the ethanol concentration. Hence, the initial rates for each CD were plotted against the ethanol concentration (Fig. 8). The value for the α-CD was decreased appreciably with increasing ethanol concentration. This may be partly caused by the inactivation of CGTase based on the addition of ethanol. With the β-CD and γ-CD, there were smaller changes. Figure 9 shows the relationship between the concentrations of each CD after 24 and 75 h and the ethanol concentration. The α-CD concentration showed a maximum in the vicinity of the ethanol concentration of 10% (v/v). The β-CD concentration decreased monotonically and levelled off at the ethanol concentration above 10%

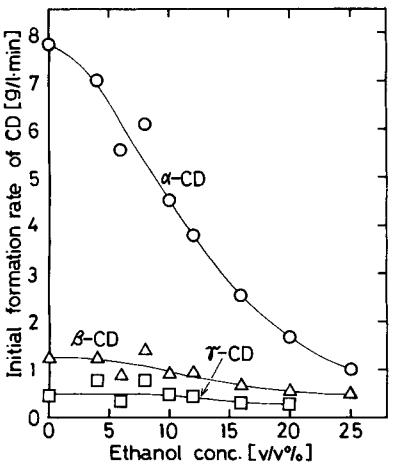


Fig. 8. Effect of ethanol concentration on initial rates of CD formation. The enzyme and initial starch concentrations are the same as in Fig. 7.

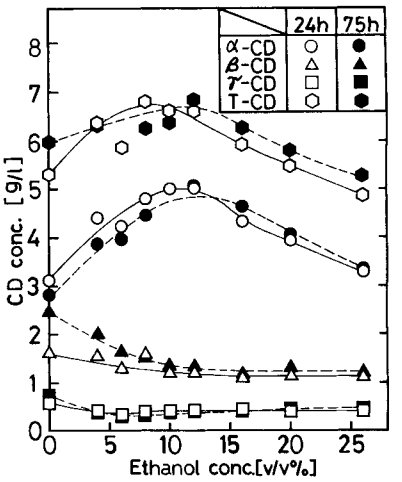


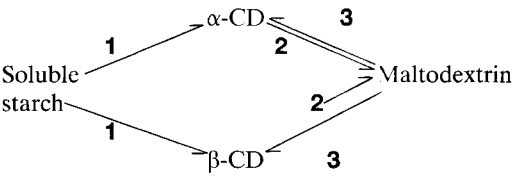
Fig. 9. Effect of ethanol concentration on CD concentration after 24 and 75 h. The enzyme and initial starch concentrations are the same as in Fig. 7.

(v/v). The change of the γ-CD concentration was small. In addition, there were no remarkable differences between the concentrations of each CD after 24 and 75 h under the reaction condition studied.

4 Discussion

When the enzyme concentration was sufficiently high, each CD was degraded almost completely after 75 h in both cases with and without ethanol (Fig. 6). The similar result has been reported by *Yagi* et al. [4], who used highly purified CGTases from three species of *Bacillus*. Based on these results, one would have a doubt whether it is appropriate to regard CGTase as a CD-synthesizing enzyme. Originally, CGTase may be an enzyme that produces CDs as intermediates in the process of the degradation of starch molecules because of high transglycosylation of the enzyme.

The time-transient behaviors of the α-CD and β-CD concentrations in the absence of ethanol (Figs. 3–6) can readily be understood according to the reaction scheme for *Bacillus macerans* CGTase proposed by *Kobayashi* et al. [5]:



That is, CGTase converts α-CD formed by **1** cyclization into maltodextrins by **2** hydrolysis or coupling. On the other hand, the enzyme again synthesizes α-CD or β-CD from maltodextrins by **3** cyclization after disproportionation. Both the rates of hydrolysis and coupling, however, are significantly low for β-CD. Thus, β-CD accumulates gradually with the passage of time.

In the region of low enzyme concentration, as a result of the addition of ethanol, the α-CD concentration increased, and the β-CD concentration decreased by the amount almost comparable to the α-CD increase. As the cause for this result, the following three possibilities may be considered [6]; (1) α-CD precipitates by forming an inclusion compound with ethanol, so that the hydrolysis or coupling is hard to occur, (2) ethanol changes the steric structure of starch molecules so as to promote

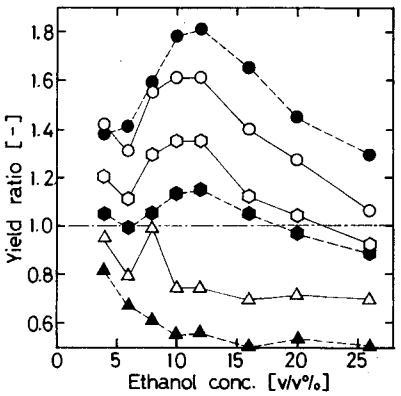


Fig. 10. Effect of ethanol concentration on yield ratio of CD formed after 24 and 75 h in the presence of ethanol to CD formed in the absence of ethanol. The enzyme and initial starch concentrations are the same as in Fig. 7 and the symbols as in Fig. 9.

α-CD formation, and (3) the formation of α-CD-ethanol complex prevents an attack of CGTase on α-CD and, as a consequence, successive conversion into β-CD is restrained.
In a preliminary experiment, we determined the solubility of the α-CD-ethanol complex to be 188 g/l at 3% (v/v) ethanol concentration and 50°C. Based on this value, we could exclude the first possibility immediately. Although what extent the second possibility concerns is difficult to estimate from only the present data, it is obvious that this explanation cannot account lucidly for the close relation between the increase in α-CD and the decrease in β-CD. When the present data are considered including the reaction scheme for pure water system proposed by *Kobayashi* et al., we may be able to conclude that the third possibility is most reasonable. The experimental explanation for this judgment will be given in our next paper.

The extent of the change in each CD yield by the addition of ethanol was examined in terms of the yield ratio of CD formed in the presence of ethanol to CD formed in the absence of ethanol. From Fig. 10, it is apparent that both yields of α-CD and T-CD were increased, while the β-CD yield was decreased in the presence of ethanol. In particular, the α-CD yield was increased significantly and gave the yield ratio of 1.8–1.9 in maximum, showing that the addition of ethanol is very effective in the production of α-CD from starch by CGTase.

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New Isomerization Technology for High Fructose Syrup Production*

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A modern glucose isomerization system has been developed which differs from conventional immobilized systems which use isomerases such as crosslinked cell-based products; the latter are discarded after enzyme inactivation. The newer system consists of two separate phases: an inert, regenerable carrier that has a high protein binding capacity, and an enzyme solution containing a pure isomerase protein. This system offers different possibilities for the immobilization:
Batch immobilization loading the carrier fully with the enzyme.
Batch immobilization loading the carrier only partly with the enzyme.
Continuous or semi-continuous loading of the carrier with enzyme during the isomerization process.
The technique of a gradual addition of enzyme to the column during the isomerization process, which is called „on-column loading", is a

Neue Isomerisierungs-Technologie für die Herstellung fructosereicher Sirupe. Ein modernes Glucoseisomerisierungs-System wurde entwickelt, welches sich von konventionellen immobilisierten Systemen unterscheidet, die Isomerasen als vernetzte zellfixierte Produkte verwenden. Letztere werden nach der Enzyminaktivierung verworfen. Das neuere System besteht aus zwei Phasen: einem inerten regenerierbaren Träger mit hohem Eiweiß-Bindungsvermögen und einer Enzymlösung, die ein reines Isomerase-Protein enthält. Dieses System bietet verschiedene Möglichkeiten der Immobilisierung:
Chargen-Immobilisierung durch volle Enzymbeladung des Trägers.
Chargen-Immobilisierung durch nur teilweise Enzymbeladung des Trägers.
Kontinuierliche oder halbkontinuierliche Beladung des Trägers mit Enzym während des Isomerisierungsprozesses.