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# Study of the action of human salivary alpha-amylase on 2-chloro-4-nitrophenyl α-maltotrioside in the presence of potassium thiocyanate

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**Abstract**

The degradation mechanism of a synthetic substrate, 2-chloro-4-nitrophenyl α-maltotrioside (CNP-$G_3$), by human salivary alpha-amylase (HSA) was investigated by kinetic and product analyses. It was observed that the enzyme attacked the various CNP-maltooligosaccharides (CNP-$G_3$ to CNP-$G_6$) releasing free CNP. Addition of 500 mM potassium thiocyanate (KSCN) was also found to greatly increase the rates of CNP-release. It was the fastest with CNP-$G_3$, and, in the presence of KSCN, was almost comparable to that of degradation of maltopentaose ($G_5$). On the other hand, addition of KSCN decreased the rate of cleavage between glucan–glucan bonds in maltopentaose. Product analysis showed that KSCN addition altered the cleavage distribution which occurred 100% at the bond between CNP and $G_3$, and that product distribution of free CNP was largely dependent on substrate concentration. Formation of CNP-$G_6$, a larger product than the original substrate CNP-$G_3$, was found to be present in the digest at high concentrations of substrate and in the presence of KSCN. Based on these results, a degradation pathway for CNP-$G_3$ involving transglycosylation besides direct hydrolysis is proposed. The increase of the CNP-release by the addition of KSCN would result from a corresponding increase in the interaction between the CNP moiety and the corresponding subsite near the catalytic site, as well as the enhancement of the catalytic efficiency. © 1997 Elsevier Science Ltd.

*Keywords:* Alpha-amylase; Human salivary; 2-Chloro-4-nitrophenyl α-maltotrioside; Transglycosylation; Clinical assay

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## 1. Introduction

Synthetic substrates such as *p*-nitrophenyl glycosides are useful for the assay of glycosidases as well as amylases, because of the simplicity of the procedure involved and the low value of the blank. Thus, several chromophore-substituted maltooligosaccharides have been developed for use in the clinical assay of amylase activity in human serum and urine [1–9].

Recently, a new substrate for clinical assay, 2-chloro-4-nitrophenyl α-maltotrioside (CNP-$G_3$), has been synthesized and its assay method has been developed by Winn-Deen et al. [10]. This substrate, which is substituted at the reducing end, allows the determination of the activity of alpha-amylase without the use of helper enzymes. The reaction yields free 2-chloro-4-nitrophenol (CNP), which can be directly monitored at 405 nm. Addition of sodium azide has also been reported to increase the rate of CNP-release. A reaction scheme based on the CNP-derivative of product has been proposed as:

$$5\text{CNP-G}_3 \rightarrow 3\text{CNP} + 3\text{G}_3 + 2\text{CNP-G}_2 + 2\text{G} \qquad (1)$$

The above reaction scheme only describes multi-sites of the splitting in the substrate upon hydrolysis, and it cannot explain the whole reaction with CNP-$G_3$, especially the accumulation of CNP-$G_4$, a longer oligosaccharide than the original substrate of CNP-$G_3$. The product has been detected in the digests by human pancreatic and salivary alpha-amylases (HPA and HSA) [10]. We suggest that the accumulation implies a participation of synthetic reactions such as transglycosylation [11–13], so that another reaction scheme involving transglycosylation besides hydrolysis is required to explain the degradation of the substrate.

X-Ray crystallographic analysis has been developed recently for several amylases and the 3D-structures of the active site, comprising several tandem subsites and possible catalytic residues, have been elucidated at high resolution [14–21]. For full understanding of the reaction mechanism, an evaluation of subsite affinity by subsite theory [22–26] is also be required, which will also allow the investigation of both the cleavage patterns of labeled oligosaccharides and kinetic parameters [26–28].

This study therefore aims to investigate the reaction of CNP-$G_3$ with HSA in order to elucidate its degradation pathway by transglycosylation, and also aims to determine the effects of KSCN as a stimulant.

## 2. Experimental

*Materials.*—CNP-$G_3$ was obtained from Oriental Yeast Co. Ltd., Tokyo, and the other CNP-glycosides (CNP-$G_6$, CNP-$G_4$, CNP-$G_2$, and CNP-G) were obtained from Genzyme Co., Kent. Soluble starch was a product of Wako Pure Chemical Industries, Ltd., Osaka, and maltopentaose was obtained from Nacalai Tesque Inc., Kyoto. *p*-Nitrophenyl α-maltotrioside (PNP-$G_3$) was purchased from Boehringer Mannheim GmbH. The enzymes used, HPA and HSA, were purchased from Aalto Scientific Ltd., CA and Sigma Chemical Co., respectively. HSA was subjected to gel filtration on a Cellulofine GCL-2000-sf column (Seikagaku Kogyo Co., Ltd., Tokyo), previously equilibrated with 5 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0), containing 5 mM $Ca(OAc)_2$ and 0.02% $NaN_3$. The second active peak (lower molecular weight fraction, 410 U/mg) was collected and stored at 4 °C prior to analysis. The sample was free from alpha-glucosidase when it was assayed with PNP-α-glucoside as substrate.

*Assay.*—Activity of the CNP-release was assayed, according to the method of Winn-Deen et al. [10], using KSCN instead of $NaN_3$ as the stimulant [29]. Liberation of CNP from CNP-$G_3$ was monitored with a UV recording spectrophotometer (Shimadzu UV265) under temperature-controlled conditions (37 °C). A typical reaction mixture consists of 4.55 mM CNP-$G_3$ and 0.11 unit of HSA in 1 mL of 20 mM MES buffer (pH 6.0), containing 5 mM $CaCl_2$, 51.5 mM NaCl, and 500 mM KSCN. The reaction was started by the addition of a small aliquot (50 μL) of the enzyme soln to the substrate soln pre-incubated in a cuvette. The amount of CNP released was determined by the increase in absorbance at 405 nm, using the molecular extinction coefficient value of 12,700 $M^{-1}\,cm^{-1}$, at pH 6.0.

Amylase activity was assayed by measuring the reducing sugar produced from 0.5% soluble starch by a modified Somogyi–Nelson method [30], using D-glucose as a standard. The reaction was performed in the above buffer (pH 6.0) at 37 °C. One unit of enzyme activity was defined as the amount of enzyme needed to produce 1 μmol of reducing sugar equivalent to glucose per min under the above conditions.

*Product analysis by HPLC.*—A typical analysis for the cleavage pattern of CNP-maltooligosaccharide by HSA was performed as follows: a mixture of 8 mM CNP-$G_3$ and 1.1 unit of HSA in 200 μL of the above buffer was incubated for 10 min at 37 °C. The

mixture was boiled for 3 min to stop the reaction within ca. 40% degradation. It was necessary to minimize the effect of subsequent hydrolysis of products. After centrifugation, the mixture was subjected to HPLC analysis, using a packed column of LiChrosphere RP8-5 (E. Merck) and 10% aq MeOH containing 0.02% $NaN_3$ as eluent. The eluate, at 35 °C at a flow rate of 1.25 mL/min, was monitored by a UV detector at 350 nm. Peaks were identified by comparison with authentic samples. Product distribution (molar ratio) was quantified from the peak areas of free CNP and CNP-maltooligosaccharides (area ratio: 1 for CNP, 0.377 for CNP-oligosaccharides) on the chromatogram. The cleavage frequency was calculated from the product distributions at a low substrate concentration (0.15 mM).

## 3. Results

*Time courses of CNP-release from various CNP-oligosaccharides in the absence and presence of KSCN.*—CNP-$G_3$, an α-glycoside of maltotriose, can be degraded rapidly by HSA, especially in the presence of sodium azide or KSCN [10,29]. The time courses of CNP-release from 0.15 mM CNP-oligosaccharides (CNP-$G_3$, CNP-$G_4$, and CNP-$G_6$) by HSA is shown in Fig. 1. CNP-$G_3$, the shortest substrate, was found to have the highest rate of CNP-release regardless of the KSCN addition. A time course at 4.55 mM CNP-$G_3$, the standard condition [10], showed a more prolonged linear line until over 2 of the absorbance at 405 nm (data not shown).

Addition of 500 mM KSCN resulted in an increase in the rate of aglycone-release by about 20 times at 0.15 mM CNP-$G_3$ and about 10 times at 4.55 mM. At the latter concentration, the rate of CNP-release became ca. 75% of the rate of hydrolysis at 0.5% soluble starch.

The behavior of the rate of CNP-release of the other CNP-oligosaccharides upon addition of KSCN is shown in Fig. 1. The rates of CNP-release from CNP-$G_4$ and CNP-$G_6$ at 0.15 mM were also increased by about 20 times. The rates with the three CNP-oligosaccharides were in the order: CNP-$G_3$ > CNP-$G_4$ > CNP-$G_6$. With CNP-$G_6$, a little lag was observed at the beginning of the reaction. The rate with CNP-$G_2$ was about ten times slower than with CNP-$G_3$ (data not shown).

*Effect of KSCN addition on kinetic parameters.*—As the addition of 500 mM KSCN prominently increased the rate of CNP-release from CNP-$G_3$ by

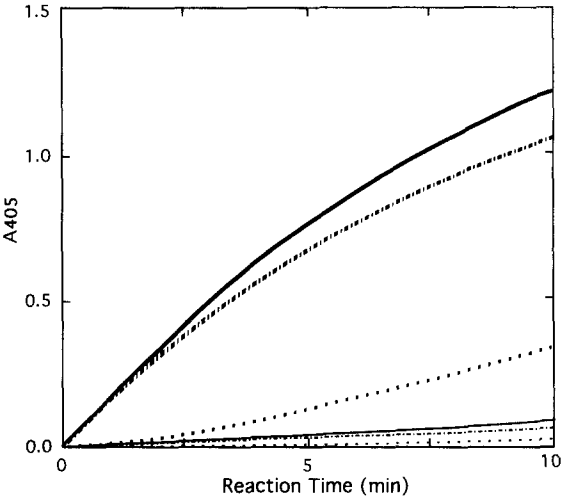


Fig. 1. Time courses of aglycone-release from CNP-maltooligosaccharides (0.15 mM) with HSA (0.11 unit/mL) and the stimulation by the KSCN addition. The enzyme reactions were performed in a temperature-controlled cuvette cell, at 37 °C in 20 mM MES buffer, pH 6.0, containing 5 mM $CaCl_2$, 51.5 mM NaCl: (————), CNP-$G_3$; (– · – · –), CNP-$G_4$; (- - -), CNP-$G_6$. Thick lines represent the course in the presence of 500 mM KSCN and thin lines the course in its absence.

HSA, its effect on kinetic parameters was studied. The kinetic parameters of HSA for CNP-$G_3$, PNP-$G_3$ (*p*-nitrophenyl α-maltotriose), and $G_5$ (maltopentaose) are shown in Table 1. For CNP-$G_3$, the addition of KSCN decreased the $K_m$ from 2.97 to 0.92 mM, but increased the $V_{max}$ from 16% to 100%. Addition of KSCN also increased the rate of PNP-release from PNP-$G_3$ as indicated by an increase in $V_{max}$ by over 30-fold (Table 1).

The effect of KSCN on the reaction with maltopentaose was also investigated. The KSCN addition did not stimulate the rate of hydrolysis of the glucan–glucan bond but rather depressed the rate ($V_{max}$) by about half (Table 1).

*Inhibition of CNP - release from CNP - $G_3$ by $G_5$.*—An inhibition experiment of CNP-$G_3$ by $G_5$ was performed in the presence of KSCN. Although

Table 1
Kinetic parameters of HSA for CNP-$G_3$, PNP-$G_3$, and $G_5$

| | $K_m$ (mM) | | $V_{max}$ (%) [a] | |
|---|---|---|---|---|
| KSCN | 0 mM | 500 mM | 0 mM | 500 mM |
| CNP-$G_3$ | 2.97 | 0.92 | 16 | 100 |
| PNP-$G_3$ | 2.24 | 6.13 | 0.3 | 10 |
| $G_5$ | 1.00 | 1.44 | 149 | 72 |

[a] Relative value ($V_{max}$ of CNP-$G_3$ in the presence of KSCN = 100%).

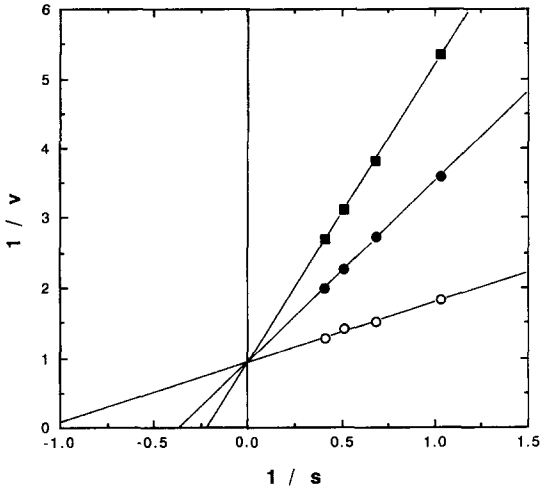


Fig. 2. A plot of 1/v versus 1/s for a competitive inhibition by maltopentaose (○, 0 mM; ●, 0.95 mM; ■, 1.95 mM) in the reaction of HSA (0.11 unit/mL) with CNP-$G_3$. The inhibition experiments were performed at 37 °C in 20 mM MES buffer, pH 6.0, containing 5 mM $CaCl_2$, 51.5 mM NaCl, and 500 mM KSCN. The rates of the CNP-release from CNP-$G_3$ at various concentrations (1.0, 1.5, 2.0, and 2.5 mM) were measured at 405 nm.

HSA attacked both substrates, CNP-$G_3$ and $G_5$, the degradation of CNP-$G_3$ could be monitored by an increase in the absorbance of free CNP at 405 nm. As shown in Fig. 2, the rate of CNP-release was inhibited by the addition of $G_5$. The common intercept on the abscissa axis in the double-reciprocal plot indicates that the inhibition was of the competitive type. Consequently, the reaction of CNP-release is likely to occur on the same active site as the reaction of bond-cleavage between glucan–glucan bonds in maltooligosaccharides. The remaining $G_5$ over the reaction period was confirmed by TLC.

*Product analysis of CNP-$G_3$ in the absence and presence of KSCN.*—Product analysis was carried out for the reaction with CNP-$G_3$ by HPLC on a reversed type column (LiChrosphere RP8-5). The chromatogram of a standard mixture of free CNP and CNP-oligosaccharides is shown in Fig. 3. The oligomers were eluted in the following order: free CNP, CNP-$G_6$, CNP-$G_4$, CNP-G, CNP-$G_3$, and CNP-$G_2$. The peak of CNP-$G_5$, which was prepared by phosphorylase reaction, was located between the peaks of CNP-$G_4$ and CNP-$G_6$ (data not shown).

The chromatogram of the digests of 8.0 mM CNP-$G_3$ in the presence and absence of KSCN are further shown in Fig. 3. Longer products, CNP-$G_4$ and CNP-$G_6$, were also detected. The amount of CNP-$G_6$ formed was found to increase in the presence of KSCN. HPLC analysis using a column of YMC AQ304, which is suitable to determine anomer of maltooligosaccharides [31,32], has revealed that the $G_3$ and $G_4$ produced were dominantly of $\alpha$-anomeric configuration (data not shown). The presence of these longer oligomers was also observed at 4.55 mM CNP-$G_3$, although the amounts were less than those at 8.0 mM (data not shown). The appearance of such products suggests synthetic activities of the enzyme, i.e. transglycosylation and/or condensation [26,28,33]. Since synthetic reactions of amylase are dependent on substrate concentration [26,28,34], digests at various substrate concentrations (0.15–8 mM) were analyzed.

The distribution of the products at various concentrations of CNP-$G_3$ in the presence and absence of KSCN is shown in Fig. 4. In the absence of KSCN, the distribution of CNP-G was held constant irrespective of the substrate concentration. As for CNP-$G_2$, the distribution was observed with an increase in substrate concentration. These results suggest that

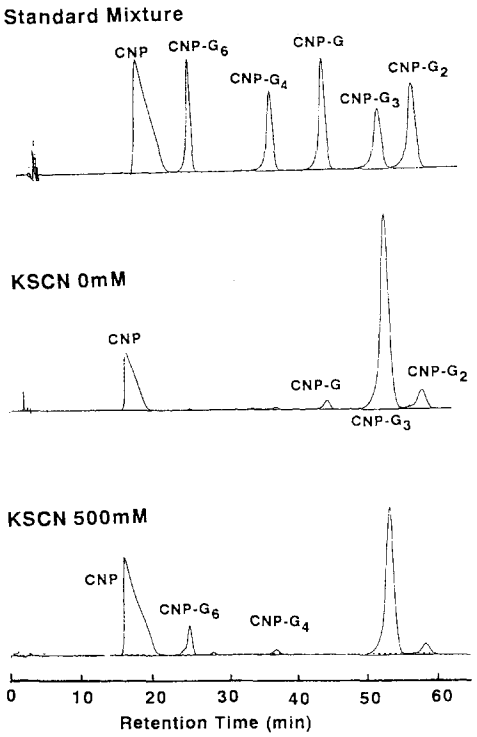


Fig. 3. HPLC analyses of the digests of CNP-$G_3$ (8.0 mM) with HSA (1.1 unit/mL) in the absence (middle) and presence of 500 mM KSCN (lower). The reaction was performed at 37 °C for 25 min (middle) or 10 min (lower). The digests were analyzed by HPLC (LiChrosphere RP8-5; eluent, 10% aq MeOH; flow rate, 1.25 mL/min), using a UV detector at 350 nm to monitor both free CNP and CNP-oligosaccharides. Upper: a standard mixture of CNP-oligosaccharides; middle: digest in the absence of KSCN; lower: digest in the presence of KSCN.

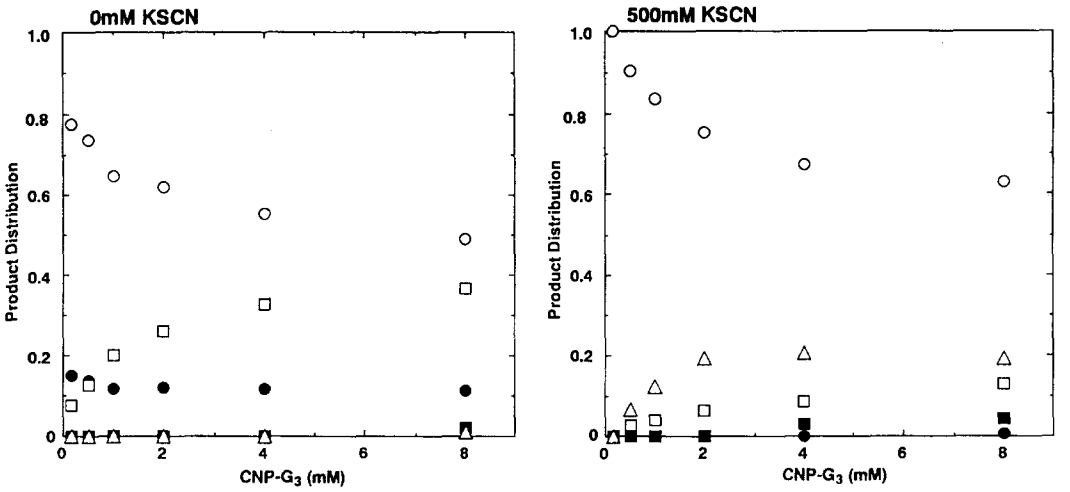


Fig. 4. The effect of the substrate concentration of CNP-$G_3$ on the product distribution of CNP-derivatives in the absence (left) and in the presence of 500 mM KSCN (right). The product distribution was calculated by a CNP-derivative (mol) divided by the sum of the CNP-derivatives (mol) in the products: ○, CNP; ●, CNP-G; □, CNP-$G_2$; ■, CNP-$G_4$; △, CNP-$G_6$.

CNP-G was produced directly through hydrolysis, while CNP-$G_2$ may be produced via some intermediate of transglycosylation, as will be discussed later.

In the presence of KSCN and with an increase in substrate concentration, distributions of CNP-$G_2$ and CNP-$G_6$ increased while that of free CNP decreased from 1.0 to 0.6. This implies that, at low substrate concentrations, the enzyme attacks the substrate to release free CNP alone, whereas at higher concentrations it could be possible that the other products are produced by other reactions aside from hydrolysis.

*Cleavage patterns at low substrate concentrations.* —In the reaction of amylase at low substrate concentrations, the hydrolytic reaction alone is known to be operative while synthetic reactions are negligible [26,33]. In this study, free CNP (77%), CNP-G (15%), and CNP-$G_2$ (8%) were detected by a UV monitor in the digest of 0.15 mM CNP-$G_3$ in the absence of KSCN. This suggests that the enzyme attacked more than one bond of the substrate (multi-splitting site), and the hydrolytic cleavage occurred at the bond between $G_3$ and aglycone CNP in 77% frequency (Fig. 5). In the presence of KSCN, wherein only free CNP were detected, the enzyme exclusively cleaved the bond between $G_3$ and CNP in 100% frequency (Fig. 5). With CNP-$G_3$, the most susceptible bond for enzyme action was found to be the bond between $G_3$ and CNP, irrespective of the addition of KSCN.

With CNP-$G_4$, the most susceptible bond was that between $G_4$ and CNP in 75% and that between $G_2$ and CNP-$G_2$ in 80% frequency, in the presence and absence of KSCN, respectively (Fig. 5). With CNP-$G_6$, the most susceptible bond was found to be that between $G_4$ and CNP-$G_2$, regardless of KSCN addition. The cleavage to release free CNP occurred only

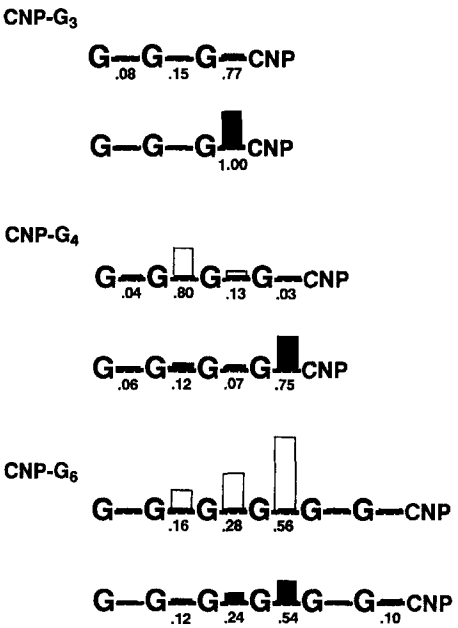


Fig. 5. Histograms of the cleavage distributions of CNP-maltooligosaccharides at low concentration (0.15 mM) in the absence (white columns) and in the presence (black columns) of 500 mM KSCN with HSA. Data below the glycosidic bond represent the cleavage frequencies calculated from the product distributions at low concentrations of substrates. The height of the column represents the relative rate of the cleavage to that of the CNP-release from CNP-$G_3$.

Table 2
The cleavage distribution of CNP-$G_3$ and CNP-$G_4$ by HPA

| | |
|---|---|
| 0.15 mM CNP-$G_3$ | G—G—G—CNP |
| −KSCN | 0.08 0.04 0.88 |
| +KSCN | 0.04 0.00 0.96 |
| 0.15 mM CNP-$G_4$ | G—G—G—G—CNP |
| −KSCN | 0.06 0.55 0.30 0.09 |
| +KSCN | 0.07 0.14 0.15 0.64 |

Data below the glycosidic bond represent the cleavage frequencies calculated from the product distributions at low concentration of substrates.

in 10% frequency, even in the presence of KSCN (Fig. 5).

*Pancreatic alpha-amylase.*—Human pancreatic alpha-amylase (HPA) was also studied with reference to its rate of CNP-release and cleavage patterns (Table 2). The rate of CNP-release at 0.15 mM CNP-$G_3$ was increased by the addition of KSCN by ca. 7 times, which was less when compared with that of HSA. As with HSA, HPA also attacked CNP-$G_3$, resulting mainly in the cleavage of the bond between $G_3$ and CNP. Addition of KSCN was also found to increase the relative frequency of bond-cleavage to release free CNP from 88% to 96% with CNP-$G_3$, and from 9% to 64% with CNP-$G_4$.

## 4. Discussion

*Reaction scheme involving transglycosylation.*—The presence of oligosaccharides longer than the substrate, such as CNP-$G_4$ and CNP-$G_6$ in the enzymic digest of 4.55 mM CNP-$G_3$, suggests participation of synthetic reactions. Human alpha-amylases are known to have both hydrolysis and transglycosylation activities [11–13]. Since no condensation can occur in the case of synthetic substrates substituted at the reducing end, the transfer reaction alone should be taken into account.

The active site of HSA is assumed to comprise five subsites with the catalytic site located between subsites 3 and 4, as indicated by product analysis [35] and X-ray analysis of animal alpha-amylases [15,17].

The cleavage frequency can be determined from the product distribution at low substrate concentration as described previously. In the case of maltooligosaccharides substituted by an aglycone at the reducing end, the cleavage frequency can be calculated from the product distribution of the substitute (aglycone) and the substituted oligosaccharides produced by the corresponding cleavage [22,23]. In this study, HPLC analysis revealed that free CNP was detected as a sole product with a UV monitor in the reaction with 0.15 mM CNP-$G_3$. This indicates that, in the presence of KSCN, the enzyme only cleaved the bond between $G_3$ and CNP at 100% frequency (Fig. 5).

From these results, a degradation pathway of 4.55 mM CNP-$G_3$ in the presence of KSCN is proposed as shown in Fig. 6. At first, the CNP-$G_3$ molecule is bound to the active site of the enzyme in such a way in 100% frequency as to form a maltotriosyl–enzyme complex upon the release of a free CNP. The major fraction of the complex then reacts with a water molecule to release a $G_3$ molecule (hydrolysis). As an alternative pathway, the remaining, minor fraction of the complex reacts with another substrate molecule,

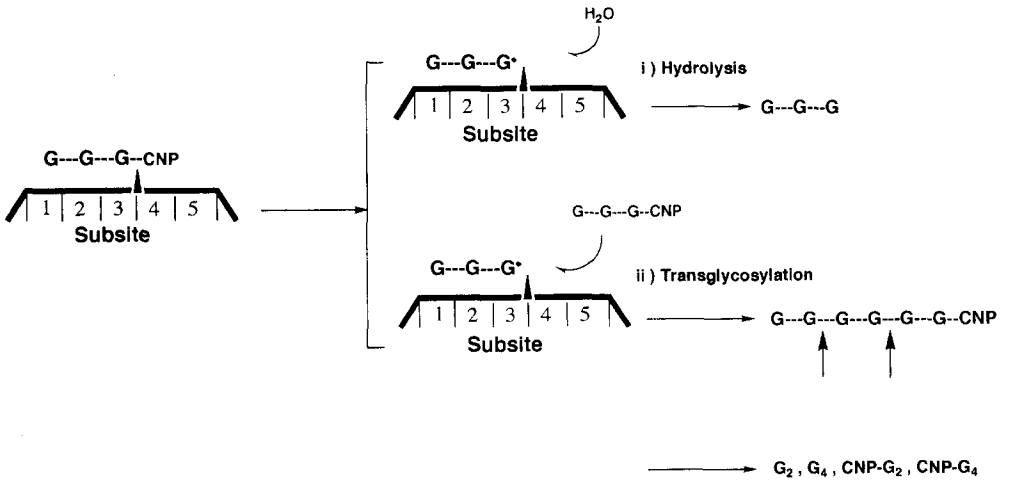


Fig. 6. Scheme for the degradation pathway of CNP-$G_3$ involving transglycosylation besides direct hydrolysis by HSA in the presence of 500 mM KSCN. Numerals represent the subsite number, numbered from the non-reducing end, and the wedge shows the catalytic site of the enzyme. The G—G—G˙ represents the active intermediate. The arrow (↑) indicates the splitting site of CNP-$G_6$ by the subsequent hydrolysis.

instead of a water molecule, to form a CNP-$G_6$ molecule (transglycosylation). The CNP-$G_6$ would then be rapidly hydrolyzed into mainly $G_4$ and CNP-$G_2$, or $G_3$ and CNP-$G_3$.

The greater accumulation of CNP-$G_6$ in the presence of KSCN (Figs. 3 and 4) occurred as a result of an increase in the rate of transfer action to form CNP-$G_6$ and a decrease in the rate of the subsequent hydrolysis between glucan–glucan bonds in CNP-$G_6$ (Table 1).

A sigmoidal time course of CNP-release appeared in the reaction of HSA with CNP-$G_6$ and the rate was much slower than in the reaction with CNP-$G_3$ and CNP-$G_4$ (Fig. 1). These CNP-oligosaccharides can also be produced by hydrolysis of CNP-$G_6$ (Fig. 5). It has been reported that a time course of its subsequent degradation would become sigmoidal when an intermediate product is re-attacked and degraded by the enzyme more rapidly than the original substrate [28].

Winn-Deen et al. have proposed the equation for the reactions of human alpha-amylases with CNP-$G_3$ as shown in Eq. (1) [10]. The equation merely describes a reaction for multi-splitting of the substrate on hydrolysis: the cleavage between $G_3$ and CNP occurs in 60% frequency and that between G and CNP-$G_2$ in 40%. However, this equation cannot explain our findings wherein CNP-$G_6$ accumulated in the digest of CNP-$G_3$ at higher concentrations, especially in the presence of KSCN (Figs. 3 and 4). And thus, a transglycosylation pathway to form CNP-$G_6$ is required in order to explain the whole reaction. According to the proposed pathway (Fig. 6), CNP-$G_2$ would be produced through a transfer product of CNP-$G_6$, but not directly by hydrolysis. This was also supported by the fact that no significant amount of glucose was detected in the digest by HPLC (data not shown). Simple hydrolysis of the substrate would yield glucose and CNP-$G_2$ in a 1:1 molar ratio.

In the presence of KSCN, the enzyme hydrolyzed CNP-$G_3$ to produce free CNP in 100% frequency at low concentrations (Fig. 5). Since a transfer product of CNP-$G_6$, if any, could produce only a little amount of free CNP by subsequent hydrolysis, the proportion of the direct hydrolysis can then be estimated by the product distribution of the free CNP to the total CNP-derivatives (Fig. 5). For instance, at 4.55 mM the proportion can be seen to be ca 70%. Thus, ca.70% of CNP-$G_3$ will be degraded through direct hydrolysis, while the remaining part will undergo transglycosylation. However, the observed increase in the absorbance at 405 nm is likely to be caused for 100% by the release of free CNP from the substrate CNP-$G_3$ at the beginning of the reaction, since a binding mode to produce the CNP-release would occur in 100% frequency on the first step in either the hydrolysis or transglycosylation pathway (Fig. 6).

In the absence of KSCN, a binding mode to release free CNP was 77%, not 100% (Fig. 5). The reaction scheme, for degradation of CNP-$G_3$, would be virtually the same as in the presence of KSCN except for the formation of CNP-G and CNP-$G_2$ through direct hydrolysis (Figs. 4 and 5).

Some isozymes are known to be present in HSA, although no differences in their modes of action on modified PNP-oligosaccharides have been found [36,37]. Our conclusion resulting from the lower molecular weight isozyme would then be applicable to other isozymes.

It has been reported that HPA and HSA have quite similar amino acid sequences [38] and action patterns toward modified phenyl $\alpha$-maltooligosaccharides [35]. This study has also revealed that the cleavage patterns for both enzymes are essentially the same (Table 2), although the effect of KSCN addition is more pronounced in HSA than in HPA (Fig. 5). Thus, the proposed pathway for CNP-$G_3$ degradation would also be applicable to the reaction of HPA.

*On the effect of KSCN addition.*—Results have shown that the addition of KSCN increased the rate of aglycon CNP-release from CNP-maltooligosaccharides (Fig. 1). The rate with CNP-$G_3$ was the fastest, which is attributed to the decrease in the $K_m$ value and an increase in $V_{max}$. The $K_m$ value decreased from 2.97 to 0.92 mM (Table 1), which is almost as small as that for $G_5$ (1.0 or 1.4 mM). This is surprising since the glucan moieties were quite different in terms of length. In general, the $K_m$ value of alpha-amylases greatly depends on the degree of polymerization of substrate maltooligosaccharides, and shorter oligosaccharides have larger $K_m$ values [39]. This implies that, in the presence of KSCN, the affinity of the enzyme for the CNP derivative of $G_3$ was as strong as that for $G_5$. Thus, this suggests that the CNP moiety plays a role in the affinity equivalent to two glucose residues of $G_5$.

The addition of KSCN has also affected the cleavage pattern. With CNP-$G_3$, the cleavage of the bond between $G_3$ and CNP increased from 77% to 100% in frequency, while with CNP-$G_4$, the cleavage between $G_4$ and CNP increased from 3% to 75%. These results also suggest that the addition of KSCN enhances the interaction between the CNP moiety and a subsite near the catalytic site (subsite 4 or 5 in Fig. 6).

Such an increase in aglycone-release activity was also observed in the chemical modification of Taka-amylase A [40] and porcine pancreatic alpha-amylase [41–43] in their reaction with PNP-maltoside. Yamashita et al. have also reported that chemical modification of the lysine residue on PPA enhanced the $k_{cat}/K_m$ values for *p*-nitrophenyl substrates, but not for phenyl substrates [44]. Thus, it can be said that this effect is substituent (aglycone) selective. The results of this study have also shown that the addition of KSCN also increased the rate of PNP-release.

The stimulation of CNP-release by the addition of KSCN was increased with an increase in the concentration of KSCN until about 500 mM, after which it decreased beyond the concentration (data not shown). Results of the dilution experiment performed indicate that the stimulation by the KSCN addition is not caused by covalent modification. When the reaction mixture was diluted after a given reaction period, the rate of CNP-release rapidly decreased at a rate corresponding to a diluted concentration of KSCN (unpublished data).

Unlike other alpha-amylases from microbial and plant sources, animal alpha-amylase alone is activated by the chloride ion. It was also observed that the addition of KSCN increased (over ten-fold) the rate of CNP-release from CNP-$G_3$ by Taka-amylase A, a typical fungal alpha-amylase (unpublished data). Thus, the stimulation by the addition of KSCN cannot be directly associated with the activation of the chloride ion.

The mechanism involved in the stimulation by the addition of KSCN is still not fully understood. What amino acid residue(s) on the active site of the enzyme is responsible for the increase in the catalytic efficiency and/or the enhancement of the subsite affinity towards a group of CNP is still unknown. Thus, further studies regarding this mechanism are now under investigation.

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