

Inhibition, activation, and stabilization of α -amylase family enzymes

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Abstract: Acarbose is a well-known inhibitor of α -glucosidases, α -amylases, cyclomaltodextrin glucanyltransferase (CGTase), and dextransucrase. *Bacillus stearothermophilus* maltogenic amylase (BSMA) was found to hydrolyze acarbose to give acarviosine-glucose plus glucose and to carry out a transglycosylation reaction to give isoacarbose. Acarviosine-glucose was a potent inhibitor of baker's yeast α -glucosidase, 430-times better than acarbose; isoacarbose inhibited porcine pancreatic α -amylase (PPA) 15-times better than acarbose. Many other acceptors reacted with acarbose and BSMA to give a series of acarbose analogues modified with acarviosine-glucose attached primarily to the C-6-OH of the nonreducing-end of the acceptor. Two interesting products were acarviosine-glucose attached to cellobiose and lactose. These acarbose analogues were inhibitors for β -glucosidase and β -galactosidase, with K_I values of 0.52 μ M and 159 μ M, where acarbose was not an inhibitor at all for these enzymes. Modification at the nonreducing-end of acarbose was accomplished by reacting acarbose with cyclomaltohexaose and CGTase to give maltohexaose and maltododecaose attached to the C-4-OH of acarbose (G6-Aca & G12-Aca). These analogues were very potent inhibitors of α -amylases. G6-Aca inhibited α -amylases from *Aspergillus oryzae* (AOA), *Bacillus amyloliquefaciens* (BAA), human saliva (HSA), and PPA with K_I values of 37, 33, 14, and 7 nM, respectively, and G12-Aca had K_I values of 81, 59, 18, and 11 nM, respectively, the most potent α -amylase inhibitors known. PPA lost activity exponentially over 2 hrs under optimum conditions. Addition of 0.02% (w/v) Triton X-100 gave 41% activation with stabilization. Seven polyethylene glycols (PEGs) at 0.02% with molecular weights of 400 to 8 kDa and two polyvinyl alcohols of 10 and 50 kDa also gave activation with stabilization. These additives were examined for 10 starch-degrading enzymes and found to be primarily more effective than Triton X-100. PEG 1.5 and 2 kDa gave the maximum degrees of activation for PPA, AOA, BAA, BLA, β -amylase, isoamylase, and CGTase, ranging from 20% to 77%. Triton X-100 gave maximum activation for HSA (45%) and pullulanase (27%). It is postulated that the enzymes have several tertiary structural forms that in solution are in dynamic equilibrium with each other. The additives that give maximum activation bind to the protein-enzymes to give a single, optimum structure that is fixed and gives the maximum activity with stabilization.

Key words: acarbose, acarbose analogues, reducing-end analogues, nonreducing-end analogues, inhibitors, activators, stabilizers, α -glucosidases, β -glucosidases, α -amylases, β -amylase, isoamylase, cyclomaltodextrin glucanyltransferase, glucose-sites, polyethylene glycols, polyvinyl alcohols, Triton X-100.

Abbreviations: Aca, acarbose; AOA, *Aspergillus oryzae* α -amylase; BAA, *Bacillus amyloliquefaciens* α -amylase; BLA, *Bacillus licheniformis* α -amylase; BSMA, *Bacillus stearothermophilus* maltogenic amylase; β -A, barley β -amylase; CGTase, cyclomaltodextrin glucanyltransferase; CD6, cyclomaltohexaose; GA, *Aspergillus niger* glucoamylase; G2, maltose; G6, maltohexaose; G12, maltododecaose; HSA, human salivary α -amylase; IA, *Pseudomonas amyloclavata* isoamylase; PEG, polyethylene glycol; PPA, porcine pancreatic α -amylase; PUL, *Bacillus acidopullulyticus* pullulanase; PVA, polyvinyl alcohol; TLC, thin-layer chromatography.

Synthesis of acarbose analogues at the reducing-end

Acarbose is a well-known, natural product produced by several species of *Actinoplanes*. It has been shown to be an effective inhibitor of several carbohydrases: α -glucosidase (SCHMIDT et al., 1982), glucoamylase (ALESHIN et al., 1994), cyclomaltodextrin glucanyltransferase (CGTase) (STROKOPYTOV et al., 1995), α -amylase (BRZOZOWSKI & DAVIES, 1997), and dextransucrase (KIM, et al. 1998). Acarbose is a pseu-

dotetrasaccharide that has a pseudosugar ring, [4,5,6-trihydroxy-3-(hydroxymethyl)-2-cyclohexen-1-yl] at the nonreducing-end, linked to the nitrogen of 4-amino-4,6-dideoxy-D-glucopyranose (4-amino-4-deoxy-D-quinovopyranose), which is linked α -1 \rightarrow 4 to maltose (Fig. 1).

The mechanism of inhibition for the above mentioned enzymes have been postulated to be the unsaturated cyclohexene ring and the glycosidic nitrogen, which is usually protonated to give a positively charged nitrogen atom. The structure is thought to mimic the

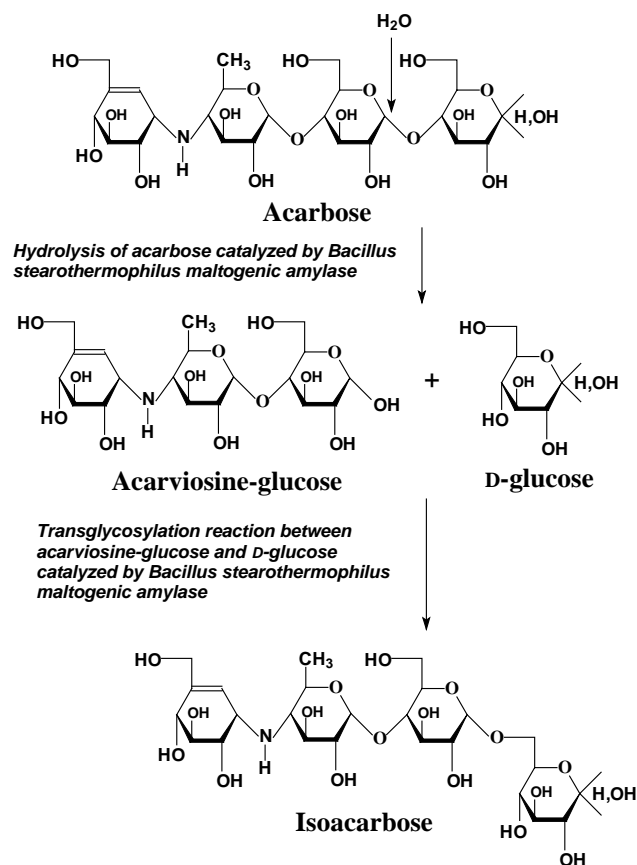


Fig. 1. Structure of acarbose and the reaction of *Bacillus stearothermophilus* maltogenic amylase with acarbose.

transition state for the cleavage of glycosidic linkages (JUNGE et al., 1980; TRUSCHEIT et al., 1981).

In 1997, Professor Kwan Hwa Park of Seoul National University found (KANG et al., 1997) that *Bacillus stearothermophilus* maltogenic amylase (BSMA) hydrolyzed acarbose at the first glycosidic linkage from the reducing-end to give D-glucose plus acarviosine-glucose. In addition, BSMA catalyzed a transglycosylation reaction between acarviosine-glucose and D-glucose to give a new pseudotetrasaccharide with acarviosine-glucose linked α -1 \rightarrow 6 to D-glucose, which is *isoacarbose* (see Fig. 1 for the reactions and structures).

Professor Park came to my laboratory on Sabbatical with this knowledge and BSMA. There we studied the scope of the transglycosylation reactions, using acarbose, BSMA, and various carbohydrate acceptors: D-glucose, D-glucitol, D-mannose, D-galactose, α -methyl-D-glucopyranoside, D-xylopyranose, D-fructopyranose, maltose, cellobiose, lactose, α - α -trehalose, sucrose, raffinose, and maltotriose. The products of the transglycosylation reactions gave acarbose analogues with acarviosine-glucose joined primarily to the C-6 position of the acceptors, and in some cases to the C-4 position, e.g., D-xylopyranose and in other cases to both the C-4 and the C-6 positions, e.g., raffinose and mal-

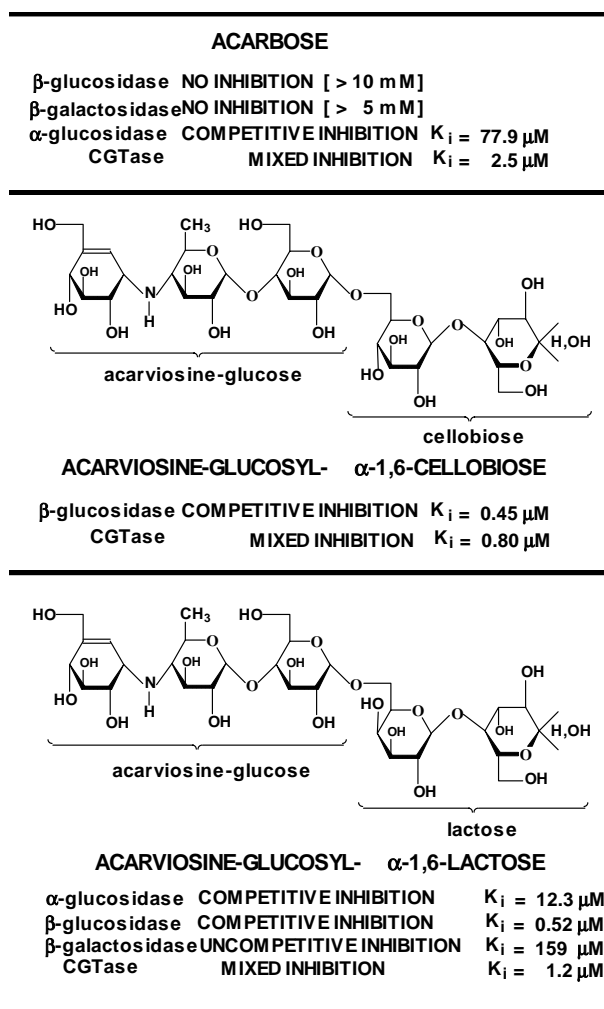


Fig. 2. Structures of acarviosine-glucosyl- α -(1 \rightarrow 6)-cellobiose and acarviosine-glucosyl- α -(1 \rightarrow 6)-lactose and their inhibition constants compared with the inhibition constants of acarbose.

totriose (PARK et al., 1998). When D-glucose was the acceptor, isoacarbose was formed.

Inhibition of glycosidases, CGTase, and α -amylases by reducing-end acarbose analogues

Acarbose, acarviosine-glucose, and isoacarbose were studied as inhibitors of α -glucosidase, α -amylase, and CGTase. Acarviosine-glucose was a potent inhibitor for baker's yeast α -glucosidase, inhibiting 430-times more than acarbose, and an excellent inhibitor of CGTase, inhibiting 6-times more than acarbose. Isoacarbose was an effective inhibitor of porcine pancreatic α -amylase and CGTase, inhibiting 15.2- and 2.0-times more than acarbose, respectively (KIM et al., 1999).

Two very interesting acarbose analogues that were synthesized resulted from cellobiose and lactose as acceptors to give acarviosine-glucose attached to the C-6 position of the nonreducing-end of these two disaccharides, giving acarviosine-glucosyl- α -1 \rightarrow 6-cellobiose

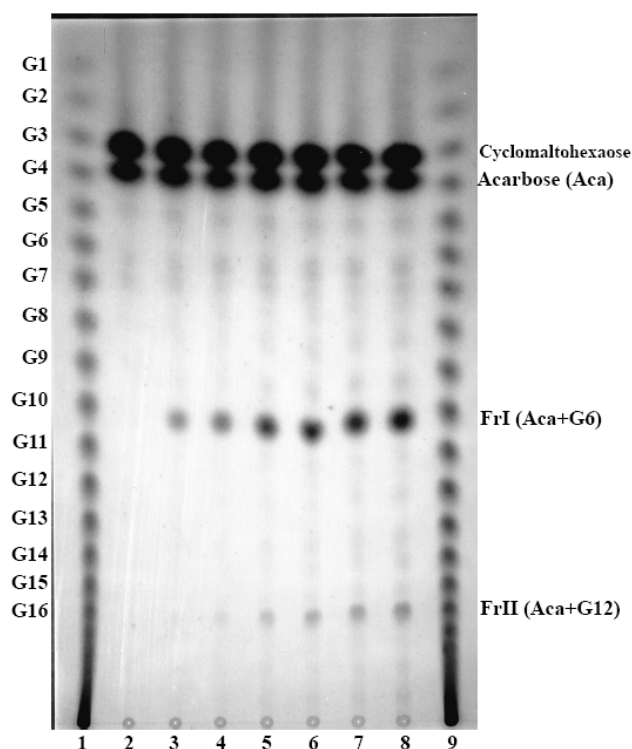


Fig. 3. Thin-layer chromatogram of the *Bacillus macerans* CGTase reaction with cyclomaltohexaose and acarbose. Whatman K5F plate was irrigated two-times for 18.0 cm each with 85:20:50:70 MeCN/EtOAc/propanol-1/water at 20°C. Carbohydrates were visualized by dipping the plate into a methanol solution, containing 0.3% (w/v) N-(1-naphthyl)ethylenediamine and 5% (v/v) sulfuric acid, followed by heating at 120°C for 10 min. Lanes 1 and 9 are maltodextrin standards; lanes 2-8 digest after 0, 0.5, 1, 2, 3, 4, and 5 days.

and acarviosine-glucosyl- α -1 \rightarrow 6-lactose (see Figure 2 for the structures and K_I values). These acarbose analogues were 6.3- and 3-times, respectively, more potent inhibitors for α -glucosidase and CGTase than was acarbose (LEE et al., 2001). In addition, they were inhibitors for β -glucosidase and β -galactosidase, with K_I values of 0.52 μ M and 159 μ M, respectively, where acarbose was not an inhibitor at all for these enzymes.

Synthesis of acarbose analogues with maltodextrins at the nonreducing-end

After making several acarbose analogues with modifications at the reducing-end, it was decided to modify acarbose at the nonreducing-end. *Bacillus macerans* CGTase has been recognized for some time to carry out transglycosylation reactions in which cyclomaltohexaose (CD6) ring is opened and maltohexaose (G6) is transferred to the C-4 position of the nonreducing-end of a carbohydrate (BENDER, 1986; YOON & ROBYT, 2002). Even though acarbose is an inhibitor of CGTase, we reasoned that maltohexaose could be added to the nonreducing-end of acarbose by CGTase by starting with a high ratio of cyclomaltohexaose to acarbose. It

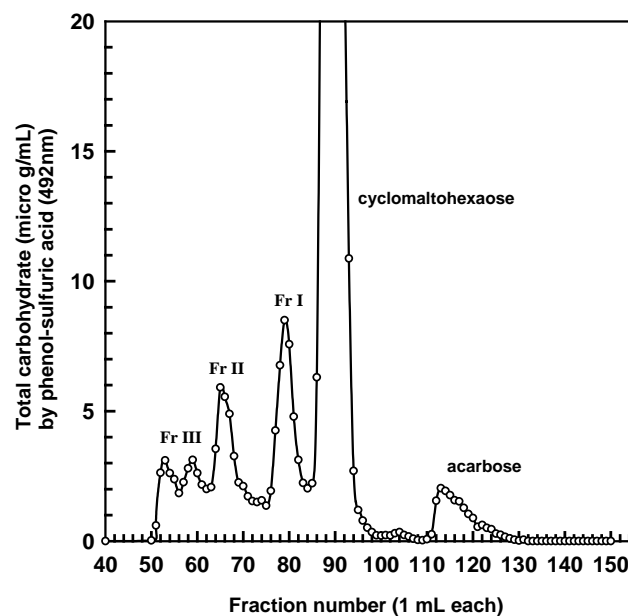


Fig. 4. Purification of the *Bacillus macerans* CGTase reaction with cyclomaltohexaose and acarbose on Bio-Gel P2 (fine) gel-permeation column (1.5 \times 100 cm); flow rate 0.06 mL/min; fraction size, 1 mL.

was further postulated that the active site of CGTase would have a higher affinity for cyclomaltohexaose than for acarbose.

Thin-layer chromatography (TLC) of the reaction digest showed that indeed a major product was formed that had a migration rate that indicated 10 monosaccharide units, a combination of maltohexaose (6 units) and acarbose (4 units), with smaller amounts of a larger saccharide containing 16 monosaccharide units, a combination of maltododecaose (G12; 12 units) and acarbose (Fig. 3). The reaction digest was purified on a Bio-Gel P2 (fine) column (1.5 \times 100 cm) by elution with deionized water (Fig. 4). Fractions I and II were reacted with β -amylase and glucoamylase. The purity of Fractions I and II and the products produced by β -amylase and glucoamylase were analyzed by TLC (Fig. 5). β -Amylase and glucoamylase are both exo-acting enzymes that produce maltose and glucose, respectively, from the nonreducing-end of a maltodextrin chain. When the two products were reacted with β -amylase, maltose (G2) and acarbose (Aca) were the main products, with a small amount of a compound indicating glucose attached to acarbose. These results demonstrate that the addition of maltodextrins were to the nonreducing-end of acarbose. Reaction of Fraction I with glucoamylase gave the formation of D-glucose and a series of G6 to G1 attached to acarbose and no acarbose (Fig. 5A, lane 5), indicating that Fraction I was G6 attached to acarbose at the nonreducing-end (G6-Aca). Reaction of Fraction II with glucoamylase gave the formation of D-glucose and a series of G12 to G1 attached to acarbose and no acarbose (Fig. 5B, lane 5), confirming that Fraction II

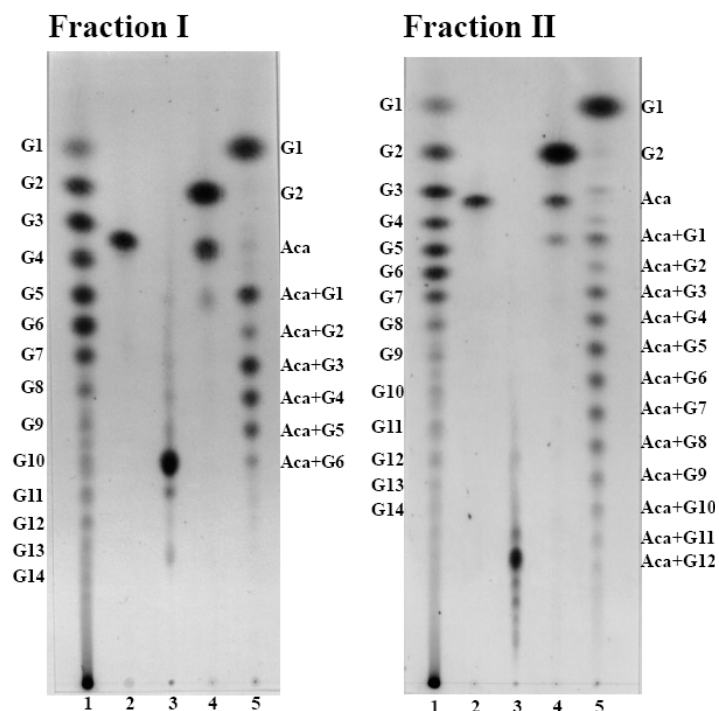


Fig. 5. Thin-layer chromatograms of purified Fractions I and II and their reactions with β -amylase and glucoamylase. Whatman K6F plates irrigated two-times for Fraction I and three-times for Fraction II for 18.0 cm each with 85:20:50:70 MeCN/EtOAc/propanol-1/water at 20°C. Carbohydrates were visualized by dipping the plate into a methanol solution, containing 0.3% (w/v) N-(1-naphthyl)ethylenediamine and 5% (v/v) sulfuric acid, followed by heating at 120°C for 10 min. Lane 1, maltodextrin standards; lane 2, acarbose standards; lane 3 purified Fractions I and II; lane 4, reaction with β -amylase; lane 5, reaction with glucoamylase.

was G12 attached to acarbose at the nonreducing-end (G12-Aca). The structures were further confirmed by two-dimensional ^{13}C -NMR (YOON & ROBYT, 2002).

The syntheses of G6-Aca and G12-Aca by the reaction of cyclomaltotetraose and acarbose with *B. macerans* CGTase are shown in Figure 6 in which cyclomaltotetraose binds in the active site of CGTase, which opens the CD6 ring, forming a covalent intermediate (LEE & ROBYT, 2001). Acarbose then comes into the active site as an acceptor, displacing maltotetraose from the active site of CGTase with its C-4-OH at the nonreducing-end, forming a covalent linkage to acarbose. G12-acarbose is formed when G6-Aca comes in as an acceptor and displaces G6 from CGTase by its nonreducing-end C-4-OH to give a linear maltododecaose chain attached to acarbose. The synthesized compounds are α -4^{IV}-maltotetraosyl acarbose (G6-Aca) and α -4^{IV}-maltododecaosyl acarbose (G12-Aca).

Inhibition of α -amylases by nonreducing-end maltodextrin analogues of acarbose

The inhibitory effects of G6-Aca and G12-Aca were examined for four α -amylases from different origins (YOON & ROBYT, 2003): fungal α -amylase from *Aspergillus oryzae* (AOA), bacterial α -amylase from *Bacillus amyloliquefaciens* (BAA), human α -amylase from saliva (HSA), and mammalian α -amylase from porcine pancreas (PPA). Amylose was the substrate used to determine the inhibition. The two inhibitors showed mixed, noncompetitive inhibition for all four of the α -amylases. Their K_I values were determined and compared with the K_I values of acarbose for each of the enzymes and are given in Table 1. Both analogues were

potent inhibitors for the four α -amylases. The K_I values for G6-Aca were 33, 37, 14, and 7 nM, respectively, for the above named four α -amylases. The K_I values for G12-Aca were 59, 81, 18, and 11 nM, respectively, for the four α -amylases. The K_I values of 7 nM and 11 nM for the inhibition of PPA by G6-Aca and G12-Aca, indicate that the two analogues are the most potent inhibitors reported for α -amylases to date. Compared with acarbose, whose K_I was 270 μM for AOA, G6-Aca was 8,182-times more potent with a K_I of 33 nM and G12-Aca was 4,576-times more potent, with a K_I of 59 nM, further showing that G6-Aca and G12-Aca analogues are the most potent inhibitors observed for α -amylases, to date, with one to three orders of magnitude more potent than acarbose, which itself is one to three orders of magnitude more potent than any of the other known α -amylase inhibitors. It is also interesting to note that the two-acarbose analogues differed in their K_I values for each of the four α -amylases and that K_I values for porcine pancreatic α -amylase was the lowest reported and therefore most potent for any known α -amylase.

PPA, HSA, AOA, and BAA are each known to have a different number of D-glucose-binding subsites at their active sites (SVENSSON et al., 2002; KANDRA et al., 2002; SABOURY, 2002). The first α -amylase to have the number of subsites determined was BAA, which was proposed to have 9 subsites from the kinds and amounts of maltodextrin products produced (ROBYT & FRENCH, 1963). This relatively large D-glucose-binding subsite was confirmed by measuring the free energy of binding of individual subsites (THOMA et al., 1970; 1971). From the action pattern of PPA, it was subsequently postulated to have 5 glucose subsites (ROBYT

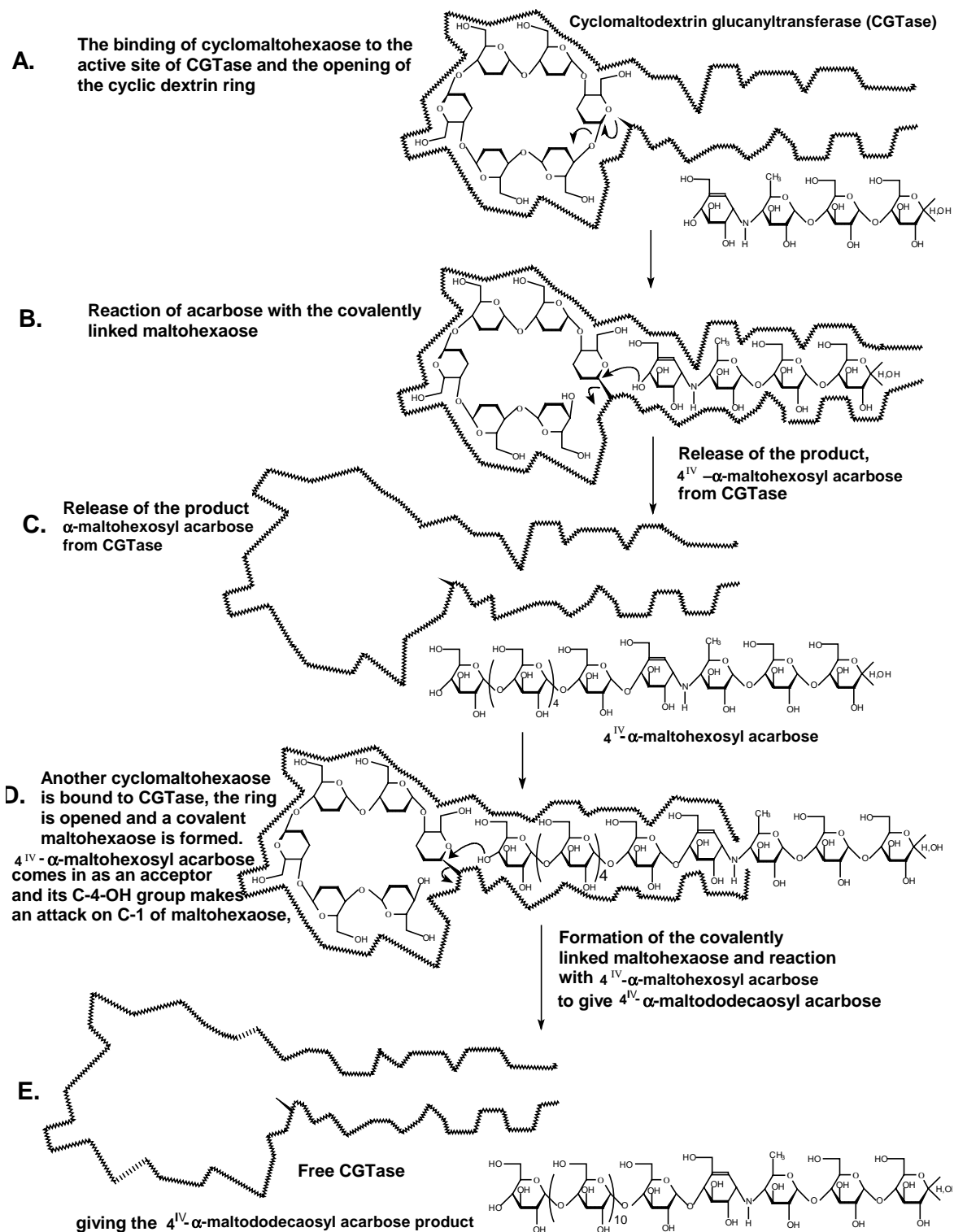


Fig. 6. Illustration of the reaction of CGTase with cyclomaltose and acarbose. (A) Cyclomaltose binds with the active site and the ring is opened and (B) a covalent intermediate is formed with maltohexaose (G6); acarbose is then bound in the acceptor site and its C-4-OH group at the nonreducing-end attacks C-1 of G6, which then becomes linked to acarbose to give (C) 4^{IV} - α -maltohexosyl acarbose (G6-Aca) and free CGTase. (D) Another cyclomaltose is bound at the active site of CGTase, the ring is opened to give the covalent maltohexaose complex and the nonreducing-end C-4-OH group of G6-Aca attacks C-1 of G6 to give (E) 4^{IV} - α -maltododecaosyl acarbose (G12-Aca).

& FRENCH, 1970). This was confirmed by determining the energy of binding glucose at the glucose-binding subsites (SEIGNER et al., 1987; DESSEAUX et al., 2002)

and by X-ray crystallographic studies, using acarbose as a substrate analogue (QIAN et al., 1994). The number of D-glucose-binding subsites of HSA was postu-

Table 1. Inhibition constants of acarbose, G6-acarbose, and G12-acarbose for four different α -amylases.

Enzyme	Inhibitors	K_I (μ M)	Inhibition potency ^a
<i>Aspergillus oryzae</i> α -amylase	Acarbose	270 \pm 39	1
	G6-Acarbose	0.033 \pm 0.003	8,182
	G12-Acarbose	0.059 \pm 0.020	4,576
<i>Bacillus amyloliquefaciens</i> α -amylase	Acarbose	13.00 \pm 3.66	1
	G6-Acarbose	0.037 \pm 0.007	351
	G12-Acarbose	0.081 \pm 0.026	160
Human salivary α -amylase	Acarbose	1.265 \pm 0.589	1
	G6-Acarbose	0.014 \pm 0.002	90
	G12-Acarbose	0.018 \pm 0.005	70
Porcine pancreatic α -amylase	Acarbose	0.797 \pm 0.156	1
	G6-Acarbose	0.007 \pm 0.002	114
	G12-Acarbose	0.0110 \pm 0.003	72

^a Inhibition potency compared to acarbose.

Table 2. Activation of ten starch degrading enzymes by 0.02% (w/v) Triton X-100, PEGs, and PVAs.^{a,b}

Additives	PPA	HSA	AOA	BAA	BLA	β -A	GA	IA	PUL	CGTase
	Relative % activity ^c									
	% SD ^d	% SD	% SD	% SD	% SD	% SD	% SD	% SD	% SD	% SD
Control	100 1.6	100 2.1	100 1.7	100 4.2	100 1.7	100 1.6	100 3.1	100 1.4	100 1.7	100 3.3
Triton X-100	141 3.0	145 3.0	132 4.1	134 2.5	135 1.5	155 4.8	118 2.8	130 3.5	127 2.3	108 0.7
PVA 10K	136 1.8	140 1.2	127 2.6	128 3.3	131 3.4	136 3.5	120 1.2	123 1.5	114 3.0	103 2.4
PVA 50K	128 5.0	142 3.0	133 1.2	121 2.2	135 2.8	121 5.7	130 3.2	121 2.0	118 3.0	102 0.4
PEG 400	100 2.5	86 4.5	134 2.4	105 1.0	119 3.7	107 4.5	103 1.2	102 0.8	104 2.2	96 3.0
PEG 600	127 1.8	133 2.0	136 1.5	116 2.3	124 4.2	119 9.4	107 4.8	100 3.8	98 1.7	101 1.2
PEG 1K	145 3.4	143 3.5	138 1.3	124 3.2	136 4.2	160 5.0	108 3.4	119 2.2	108 4.1	102 3.2
PEG 1.5K	154 2.1 ^e	139 4.1	142 4.6	123 1.8	143 3.4	177 1.7	121 6.7	132 3.6	106 0.7	120 4.3
PEG 2K	144 4.6	134 1.5	137 4.0	119 2.4	144 1.4	158 1.0	117 1.9	137 2.5	118 3.4	96 2.8
PEG 4.6K	141 3.4	129 4.8	134 2.4	119 1.1	144 3.3	174 5.2	110 2.6	136 4.5	108 0.9	91 3.9
PEG 8K	138 5.9	140 3.3	134 2.6	119 3.2	140 1.4	168 0.7	111 4.1	134 3.5	108 2.0	90 4.4

^a Abbreviations used: PPA, porcine pancreatic α -amylase; HSA, human salivary α -amylase; AOA, *Aspergillus oryzae* α -amylase; BAA, *Bacillus amyloliquefaciens* α -amylase; BLA, *Bacillus licheniformis* α -amylase; CGTase, cyclomaltodextrin glucanyltransferase; β -A, β -amylase; GA, glucoamylase; IA, isoamylase; PUL, pullulanase; PEG, polyethylene glycol; PVA, polyvinyl alcohol.

^b The activities of the α -amylases were determined using amylose as the substrate; the activities of β -amylase, glucoamylase, isoamylase, and cyclomaltodextrin glucanyltransferase were determined using waxy maize starch as the substrate; and the activity of pullulanase was determined using reduced pullulan as the substrate. From YOON & ROBYT (2005).

^c Relative percent activity = percent of activity relative to the activity of the control that had no added additives.

^d SD = standard deviation.

^e The maximum activity for each enzyme and additive is given in bold type.

lated to be 6, using maltodextrins with 2-chloro-4-nitrophenyl group at the reducing-end and maltodextrins with 4,6-O-benzilidene at the nonreducing-end (KANDRA & GYÉMÁNT, 2000; KANDRA et al., 2002). From a kinetic study of AOA, reacting with maltodextrins and the determination of the energy of binding to each subsite, it was postulated to have 7 D-glucose-binding subsites (SUGANUMA et al., 1978), and was confirmed by X-ray crystallography (MATSUURA et al., 1984; BRZOZOWSKI & DAVIES, 1997).

The X-ray crystallographic analysis of PPA in the presence of acarbose (QIAN et al., 1994) and AOA in the presence of acarbose (BRZOZOWSKI & DAVIES, 1997) showed that the two units of acarviosine at the nonreducing-end of acarbose were bound at subsites +1 and -1, where the catalytic groups were lo-

cated. These are the two units of acarbose that act as the transition-state mimics for the cleavage of the α -1 \rightarrow 4 glycosidic linkage of starch and thereby produce inhibition. On the basis of the structural features of the active site of α -amylases and related enzymes (MACGREGOR & SVENSSON, 1989; SVENSSON & SØGAARD, 1993; SVENSSON, 1994; SVENSSON et al., 2002), it can be postulated that the acarviosine unit of acarbose inhibits the α -amylases by binding with the two D-glucose-binding subsites, +1 and -1, on either side of the catalytic groups (see Figure 7, which shows G6-Aca binding at the active sites of the four α -amylases).

The study clearly shows that the attachment of maltodextrins to the nonreducing-end of acarbose greatly enhances the affinity of the acarbose unit for the

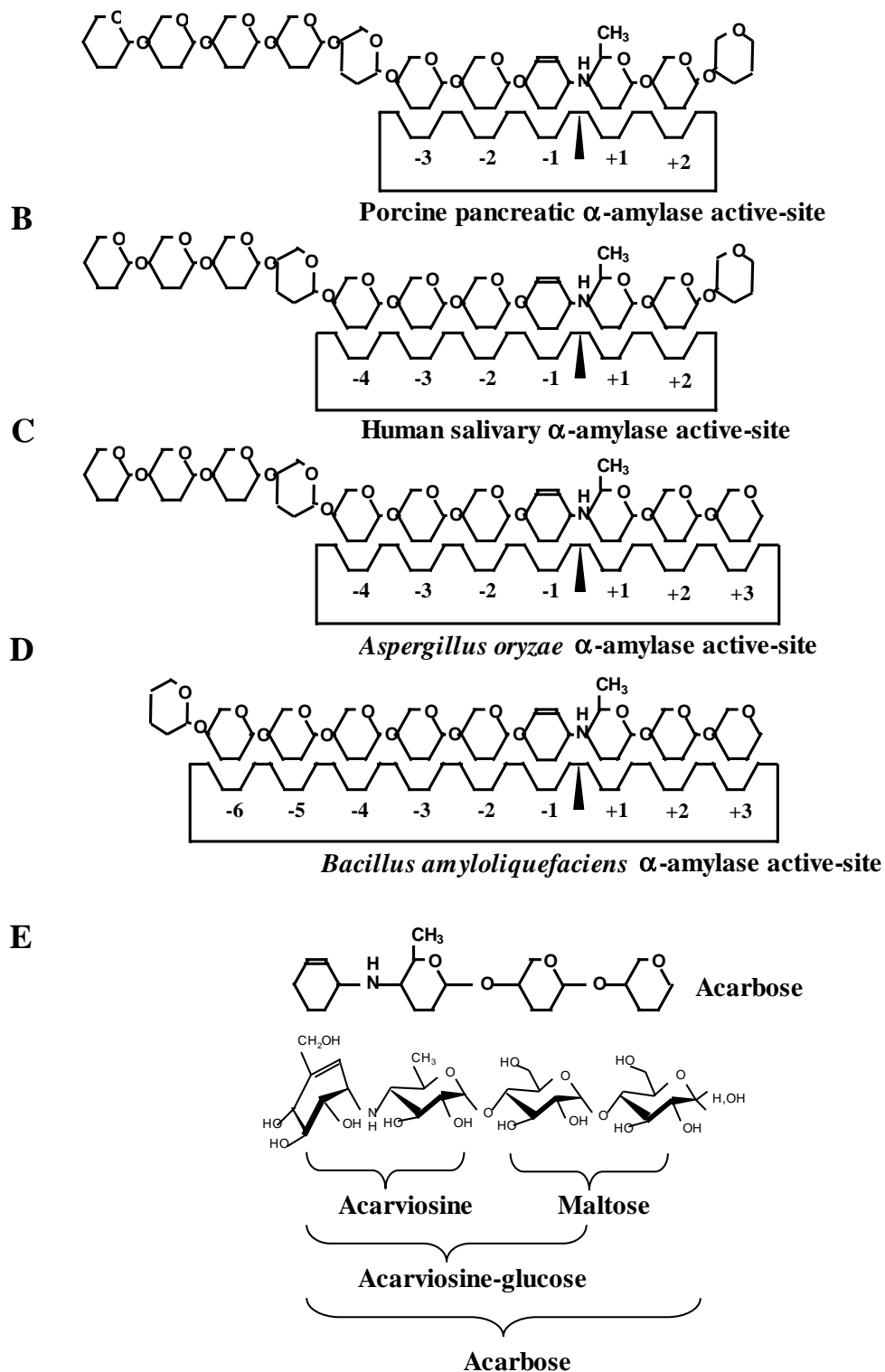


Fig. 7. D-Glucose-binding subsites for four α -amylases (A, B, C, and D) and their inhibited complexes with 4^{IV} - α -maltohexaosyl acarbose (G6-Aca). E is the structure of acarbose. The nitrogen glycosidic atom of the acarbose unit is specifically bound at the catalytic-site (represented by a black triangle), along with the two units of acarviosine (cyclohexeneitol and D-quinovose units) that are bound to the +1 and -1 subsites, respectively.

active sites of the amylases, making the analogues much more potent active site directed inhibitors than acarbose or any other known α -amylase inhibitors. Because G6-Aca and G12-Aca have relatively long maltodextrin chains, it could be expected that the α -amylases might

hydrolyze the chains. The maltodextrin-acarbose analogues were incubated with the four α -amylases and samples were taken at 0.5, 1, 2, and 3 hrs for TLC analysis. It was found that PPA, HSA, and BAA did not hydrolyze the maltodextrins over the 3 hr time period

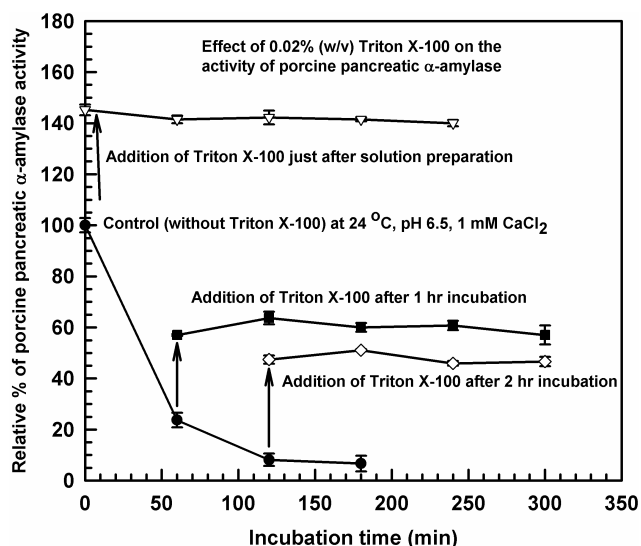


Fig. 8. Loss of porcine pancreatic α -amylase activity at pH 6.5, 24 °C, 1 mM CaCl_2 ; activation and stabilization by 0.02% (w/v) Triton X-100 and reactivation by 0.02% (w/v) Triton X-100 after loss of activity, standing 1 hr and 2 hrs.

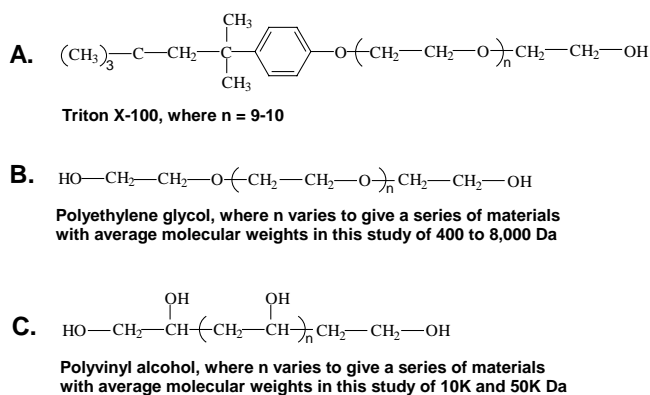


Fig. 9. Structures of (A) Triton X-100, (B) polyethylene glycols, and (C) polyvinyl alcohols.

at all and AOA produced <1% hydrolysis in 3 hrs. Apparently, the binding affinity of the acarviosine-unit of the maltodextrin-acarbose analogues to the active sites of the four α -amylases is so high that only nonproductive complexes were formed with G6-Aca and G12-Aca (YOON & ROBYT, 2003).

Activation and stabilization of amylase-family enzymes

In studying the action of porcine pancreatic α -amylase, it was observed that a dilute solution (0.01 to 10 units/mL), under optimum conditions of pH 6.5, 24 °C, and 1 mM CaCl_2 , lost all of its activity in 2 hrs (YOON & ROBYT, 2005). Addition of 0.02% (w/v) of the non-ionic detergent, Triton X-100, to a freshly prepared solution gave 45% activation and indefinite stabilization (Fig. 8).

The structure of Triton X-100 contains a polyethy-

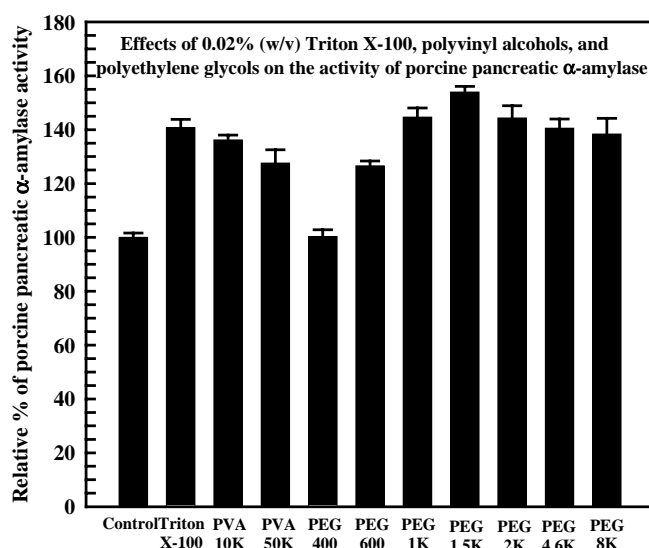


Fig. 10. Effects of adding 0.02% (w/v) Triton X-100, polyethylene glycols, and polyvinyl alcohols on the activity of porcine pancreatic α -amylase.

lene glycol chain, attached to a phenyl ring (Fig. 9). We, therefore, studied Triton X-100, seven polyethylene glycols (PEGs) with average molecular weights from 400 to 8K Da, and two polyvinyl alcohols (PVAs) of 10K and 50K average Da as activators and stabilizers of ten starch-degrading enzymes: five α -amylases - PPA, HSA, AOA, BAA, *Bacillus licheniformis* α -amylase (BLA); and five other enzymes: *Aspergillus niger* glucoamylase (GA), barley β -amylase (β -A), *Pseudomonas amyloclavata* isoamylase (IA), *Bacillus acidopullulyticus* pullulanase (PUL), and *Bacillus macerans* cyclomaltodextrin glucoamyltransferase (CGTase).

The effects of 0.02% (w/v) of the ten additives on the activation for PPA are shown in Figure 10. All of the additives gave activation, except PEG 400. The best activator for PPA was PEG 1.5K, which gave $54 \pm 2.1\%$ activation, $13 \pm 2.5\%$ greater than Triton X-100. All of the other nine enzymes gave similar results, although the different additives gave different degrees of activation and different additives gave the maximum degrees of activation, depending on the different enzymes. Table 2 gives all of the degrees of activation for the 10 additives and the 10 enzymes. PEG 1.5K gave the highest degree of activation (77%) for β -amylase. The maximum degrees of activation were primarily produced by PEG 1.5K and PEG 2K, which gave maximum degrees of activation for PPA, AOA, BAA, BLA, β -A, IA, and CGTase, ranging from 77% to 20%. Triton X-100 gave maximum degrees of activation for HSA, and PUL of 45% and 27%, respectively.

A concentration study of the activation of PPA by PEG 1.5K found that doubling the concentration to 0.04% (w/v) gave a significant increase in the degree of activation from 54% to 70%. We, therefore, doubled the concentration for the maximum activator and the first

Table 3. Comparison of the degrees of activation of the ten starch degrading enzymes at 0.02% (w/v) and 0.04% (w/v) concentrations of the additives (YOON & ROBYT, 2005).

Enzymes	Relative % activity ^a				Additives
	0.02%	(w/v)	0.04%	(w/v)	
	%	SD	%	SD	
Porcine pancreatic α -amylase	154	2.1	170	4.3	PEG 1.5K ^b
Human salivary α -amylase	145	3.0	141	3.1	Triton X-100 ^b
	143	3.5	133	0.7	PEG 1K ^c
	142	3.0	128	3.8	PVA 50K ^c
<i>Aspergillus oryzae</i> α -amylase	142	4.6	153	3.7	PEG 1.5K ^b
<i>Bacillus amyloliquefaciens</i> α -amylase	134	2.5	122	3.4	Triton X-100 ^b
	128	3.3	136	2.5	PVA 10K ^c
	124	3.2	142	4.0	PEG 1K ^c
<i>Bacillus licheniformis</i> α -amylase	144	1.4	137	0.7	PEG 2K
	144	3.3	146	2.1	PEG 4.6K
	135	2.8	140	1.9	PVA 50K
	135	1.5	139	2.2	Triton X-100
Cyclomaltodextrin glucanyltransferase	120	4.3	107	1.6	PEG 1.5K ^b
Barley β -amylase	177	1.7	135	1.8	PEG 1.5K ^b
<i>Aspergillus niger</i> glucoamylase	130	3.2	148	2.8	PVA 50K ^b
Isoamylase	137	2.5	158	4.3	PEG 2K ^b
Pullulanase	127	2.3	106	3.5	Triton X-100 ^b
	118	3.4	118	4.6	PEG 2K ^c
	118	3.0	103	0.8	PVA 50K ^c

^a Relative % activity compared with the control without any additive. Activities that increased on doubling the concentration to 0.04% (w/v) are given in bold type.

^b Additives that gave maximum activation at 0.02% (w/v) for each of the enzymes.

^c Additives that gave the next highest degrees of activation at 0.02% (w/v) for the enzymes that Triton X-100 gave the highest degrees of activation at 0.02% (w/v).

two next highest activators for each of the enzymes. For several of the enzymes the degrees of activation were decreased on doubling the concentration of the maximum activator, but in several others the degrees of activation were significantly increased (Table 3). The degree of activation of AOA was increased from $42 \pm 4.6\%$ to $53 \pm 3.7\%$ by PEG 1.5K. The degree of activation of BAA was increased from $28 \pm 3.3\%$ to $36 \pm 2.5\%$ by PVA 10K and from $24 \pm 4.0\%$ to $42 \pm 4.0\%$ by PEG 1K. The degree of activation of BLA was only slightly increased by 0.04% PEG 4.6K, PVA 50K, and Triton X-100. The degree of activation of IA was significantly increased from $37 \pm 2.5\%$ to $58 \pm 4.3\%$ by PEG 2K.

The activation of the enzymes showed specificity in that different kinds of additives and different sizes of the same additive activated to different degrees. The additives also varied as to which one gave the maximum degree of activation for the different enzymes, and there was also a concentration effect.

PEG 1.5K could activate and stabilize PPA that had lost activity, but like Triton X-100, it did not give full recovery of the activity (Fig. 8). An experiment was performed in which PEG 1.5K was added (a) only to the substrate, (b) only to the enzyme, and (c) to both the enzyme and the substrate. Addition only to the substrate gave no activation, but addition only to the enzyme and addition to both the enzyme and the substrate gave the same degree of activation. This showed that the additive is binding exclusively to the enzyme to

give the activation and that the binding is quite specific and strong so that stabilization of the activity is effective for long periods of time. Differences in the degrees of activation for some of the enzymes in which Triton X-100 was maximum and in others where different sized PEGs or PVAs were maximum, might reflect differences in the number of glucose-binding subsites at the active sites of the enzymes, e.g., the α -amylases, that give different product specificities. Another difference might be the nature of the stereochemical reactions that result in the hydrolysis of α -1 \rightarrow 4 glycosidic linkages in the case of the amylases or in the hydrolysis of α -1 \rightarrow 6 branch glycosidic linkages in the case of IA and PUL, or in the transglycosylation of α -1 \rightarrow 4 glycosidic linkages to form α -1 \rightarrow 4 cyclic maltodextrins in the case of CGTase (ROBYT, 2003).

Nine of the enzymes have the $(\beta/\alpha)_8$ -barrel motif, whose α -helical loops join the β -strands together and compose the active site clefts of the enzymes (MATSUURA et al. 1984; KUBOTA et al. 1990; KLEIN & SCHULZ 1991; MIKAMI et al. 1992; QIAN et al. 1993; MATSUURA et al. 2002; SVENSSON et al. 2002). Glucoamylase is the only exception in which an $(\alpha/\alpha)_6$ -barrel motif makes up the active site (ALESHIN et al., 1994). It was reported (SIMON et al., 1993) that α -chymotrypsin was stabilized by PEG 20K. PEG 5K was found to activate horseradish peroxidase by locking or freezing the heme active site (GUO & MABROUK, 2002) and PEG increased the activity and stabilized

enzymes when acting in solvents containing 60% organics (DABULIS & KLIBANOV, 1993). Raman spectroscopy showed that the polyol stabilizing effects on enzymes arose from direct and specific interactions with the polypeptide chain (COMBES et al., 1993).

The mechanism for the activation and stabilization of the 10 starch-degrading enzymes by Triton X-100 and the various PEGs and PVAs has been postulated (YOON & ROBYT, 2005) to occur by their hydrophobic binding to the enzyme-proteins to give an optimally folded and compact barrel-motif structure that gives the maximum amount of enzyme activity. The continued binding of the additives by a strong interaction with the enzyme-protein gives stabilization of the activity as well as activation. When no additives are present, the enzymes could have a number of structural forms that vary from a form that gives maximum activity to a form in which no activity is obtained and with any number of forms in between the two extremes. This mixture of different structural forms would give some specific amount of activity. It is postulated that the addition of the maximum activator (Triton X-100, PEG 1.5K, and so forth) would convert all of the forms, except a completely denatured form, into the structural form that has the maximum enzyme activity. It is further postulated that additives that give lower degrees of activity or even less activity (inhibition) than the form with no additive still give single structural forms that are fixed, but have less activity than the structural form that is produced by the maximum activator. In other words, we are postulating that enzymes exist in solution as a mixture of several tertiary structural forms in dynamic equilibrium, each with different amounts of enzymatic activities. The addition of the additive to the mixture shifts the equilibrium to give a single tertiary structural form that has a maximum amount of enzyme activity and by binding tightly to the enzyme gives stabilization of this activity.

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