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A novel metabolic pathway for glucose production mediated by α-glucosidase-catalyzed conversion of 1,5-anhydrofructose*

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*Running title: *α-Glucosidase-mediated novel reaction on 1,5-anhydrofructose*

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Keywords: α-glucosidase; hydration; *trans*-addition; 1,5-anhydrofructose

Capsule

Back-ground: α-Glucosidase-catalyzed hydration on 1,5-anhydrofructose has not been clarified.

Results: α-Glucosidases of glycoside hydrolase families 13 and 31 synthesize α-configurated product from 1,5-anhydrofructose. Combined reaction of glucan lyase and α-glucosidase enhances glucose-production from starch.

Conclusion: α-Glucosidases catalyze *trans*-addition on 1,5-anhydrofructose.

Significance: Novel α-glucosidase-associated metabolic pathway of glucose-formation from 1,5-anhydrofructose, suggesting a salvage process of un-utilized 1,5-anhydrofructose to glucose in nature.

SUMMARY

α-Glucosidase is in the glycoside hydrolase family 13 (13AG) and 31 (31AG). Only 31AGs can hydrate the D-glucal double bond to form α-2-deoxyglucose. Since 1,5-anhydrofructose (AF), having 2-OH-group, mimics oxocarbenium-ion transition-state, AF may be a substrate for α-glucosidases. α-Glucosidase-catalyzed hydration produced α-glucose from AF, which plateaued with time. Combined reaction with α-1,4-glucan lyase (GL) and 13AG eliminated the plateau. *Aspergillus niger* α-glucosidase (31AG), which is stable in organic solvent, produced ethyl α-glucoside from AF in 80% ethanol. The findings indicate that α-glucosidases catalyze *trans*-addition. This is the first report of α-glucosidase-associated glucose formation from AF, possibly contributing to salvage pathway of un-utilized AF.

α-Glucosidases (EC 3.2.1.20, α-D-glucosidic glucohydrolase) are retaining exo-glycosidases, which hydrolyze α-glucosidic linkage at the non-reducing end of a substrate. Various types of α-glucosidases with different substrate specificities are widely distributed in microorganisms, insect, plants, and animals (1). α-Glucosidases are mainly classified in glycoside hydrolase family (GH) 13 or 31 (1, 2).

GH 13 α-glucosidases (13AGs) (1, 3-7) have four conserved regions of α-amylase family enzymes

(8). Their three-dimensional structures have been resolved (3, 5). The catalytic domains form a $(\beta/\alpha)_8$-barrel. GH 31 α-glucosidases (31AGs) (9-14) have the conserved regions (A and B), which include the so-called catalytic nucleophile and general acid/base catalyst, respectively (13, 15). Available three-dimensional structures of 31AGs carry a catalytic $(\beta/\alpha)_8$-barrel domain (12, 13). 13AG and 31AG show clear differences in substrate recognition (1). 31AGs display hydrolysis on *p*-nitrophenyl 2-deoxyglucoside (Fig. 1A) (16) and hydration at a double bond of D-glucal (GlcAL) by *trans*-addition to produce α-2-deoxyglucose (α2dGlc; Fig. 1B) (17, 18), although 13AGs cannot catalyze those reactions (17). These findings imply that 2-OH of the glycone portion (a glucose unit of non-reducing end that binds to subsite -1) is avidly recognized by 13AGs, whereas recognition of 31AGs is not as strong.

Genes of α-1,4-glucan lyases (GL; EC 4.2.2.13), which release 1,5-anhydro-D-fructose (AF) from starch and glycogen, has been cloned and GLs were characterized (19-21). Due to a tautomerization in the aqueous solution, AF forms enol-, keto-, and hydrated-type structures (Fig. 1C) (19, 20). Although the hydrated AF is the major form in water, a 1,2-enol AF mimics the oxocarbenium-ion transition-state more than GlcAL does (22). Furthermore, 1,2-enol AF has 2-OH, which is essential for 13AG-catalyzed reaction.

In this study, we investigated the reaction of 13AG and 31AG on AF, and report that α-glucosidases catalyze hydration and 1-alcohoxy-2-hydro-addition for the first time. A combined reaction on starch with GL and 13AG enhances glucose-formation, suggesting the existence of novel metabolic pathway for glucose production.

EXPERIMENTAL PROCEDURES

Enzymes and chemicals. α-Glucosidases from *Streptococcus mutans* (DG) (4), honeybee (isozyme II; HBG2) (6), *Aspergillus niger* (ANG) (9), and buckwheat (BWG) (11), and GL from red algae (19) were purified as described previously. AF with 99% purity was produced from soluble starch by GL (20). GlcAL was prepared from the 3,4,6-tri-*O*-acetyl form (Sigma-Aldrich, St. Louis, MO, USA) (17). Ethanol was purchased from Kanto Chemical (Tokyo, Japan); soluble starch and *p*-nitrophenyl α-glucoside (*p*NPG), from Nacalai Tesque (Kyoto, Japan); conduritol B epoxide (CBE; suicide substrate for α-glucosidases), mannose and deuterium water (D_2O), from Sigma-Aldrich.

Hydration of AF by α-glucosidases. α-Glucosidase (DG, 0.46 μg/ml; HBG2, 0.40 μg/ml; ANG, 0.67 μg/ml; BWG, 0.47 μg/ml) was incubated with 30 mM AF in 20 mM sodium acetate buffer (pH 5.0) for 40 min at 35ºC [only for DG, in 20 mM sodium phosphate buffer (pH 6.0) at 25ºC]. The reaction product was analyzed by TLC with the same method of Chiba and Shimomura (23) and high-performance anion-exchange liquid chromatography (HPAEC; Dionex, Sunnyvale, CA, USA) with CarboPac PA1 (4 x 250 mm, Dionex) and a pulsed-amperometry detector (Dionex). Further analysis was done by treating the reaction product with *A. niger* glucose oxidase (Sigma-Aldrich): the reaction mixture containing 40 mM AF, 16 mM sodium acetate buffer (pH 4.0) and 2.84 μg/ml ANG was incubated at 35ºC, followed by addition of glucose oxidase (1,200 U/ml) with 0.1 M Tris-HCl buffer (pH 7.0) at 35ºC for 1 h. The reaction product was analyzed with TLC as described. Assay of glucose from AF was done using the Tris-glucose oxidase-peroxidase method (Wako, Osaka, Japan) (24, 25).

Combined reaction on starch with GL and DG. Both DG and GL (0.46 μg/ml and 0.22 μg/ml, respectively) were incubated with 2% soluble starch in 5 mM sodium phosphate buffer (pH 6.0) at 25ºC. The reaction mixture was acquired at the indicated time, and product was analyzed with methods described below. Anomeric configuration of product was determined by HPLC (JASCO, Tokyo, Japan) with TSK-GEL Amide-80 (4.6 x 250 mm; Tosoh, Tokyo, Japan) and evaporative light-scattering detector (EYELA, Tokyo, Japan).

ANG-reaction on AF in ethanol. In 80% ethanol, ANG (0.4 μg/ml) was incubated with 50 mM AF in 20 mM sodium acetate buffer (pH 4.3) at 35ºC for 2 days, followed by boiling for 10 min. A TLC-purified 1-alcohoxy-2-hydro-addition product (ethyl α-D-glucopyranoside) was analyzed using field desorption mass spectrometry (FD-MS; JEOL JMS-SX102A) and NMR (Bruker AMX-500), chemical shifts (δ) of which were expressed in ppm relative to the external standard of TSP: FD-MS, *m/z* 208; ^{1}H-NMR δ_H (500 MHz; D_2O): δ 4.92, 3.86, 3.80, 3.74, 3.58, 3.68, 3.39, 3.54, 1.23; ^{13}C-NMR δ_C (125 MHz; D_2O): δ 100.66, 72.45, 75.99, 74.04, 74.48, 63.39, 66.69, 16.89.

Inhibition with GlcAL. Inhibition of GlcAL for hydrolytic reaction on *p*NPG was analyzed using HBG2. HBG2 (1.1 μg/ml) was incubated with 100 mM GlcAL and 2.5 mM *p*NPG in 40 mM sodium acetate buffer (pH 5.0) at 37ºC for HBG2, followed by addition of 1 M Na_2CO_3. Liberated *p*-nitrophenol (*p*NP) was calculated using $\varepsilon_{1\,mM,\,400\,nm} = 5.56$.

Results and Discussion

Hirano *et al.* (26) reported that α-glucosidase could not hydrate AF, allowing us to analyze the hydrations of AF using 13AG (HBG1) and 31AG (ANG and BWG). As shown in Fig. 2A, only glucose-like monosaccharide was detected as a product in the reactions on AF with all α-glucosidases examined. The product of 13AG or 31AG was also analyzed by HPAEC, which verified glucose-formation (Fig. 2B). The product was sensitive to glucose oxidase-treatment, as evident by the absence of a glucose spot upon TLC-analysis. No product was formed by CBE-inactivated ANG, which was prepared by incubation of 2.84 μg/ml ANG with 0.1 M CBE in 20 mM sodium acetate buffer (pH 4.5) at 35ºC for 2 h (27). These results indicate that 13AG and 31AG catalyze hydration of AF to produce glucose. Since Hirano *et al.* used *N*-glycan-trimming-type α-1,3-glucosidase (a 31AG belonging to EC 3.2.1.130; glycoprotein glucosylmannohydrolase) for AF-hydration (26), it is possible that this enzyme has the different substrate specificity from 13AG and 31AG.

The time-course exhibited a plateau (Fig. 2C) at DG-catalyzed glucose-formation. AF formed keto-enol tautomers and hydrated-compound under the aqueous condition (Fig. 1C), indicating that 1,2-enol AF was a possible substrate for α-glucosidases. To prove this possibility, we performed a combined reaction on soluble starch with GL and DG (Fig. 1D). DG can act on AF but not a soluble starch, whereas the other α-glucosidases used will be able to release glucose from soluble starch (3, 4). Therefore, the formation of glucose will provide the evidence that the GL released AF from soluble starch. Generated AF can be acted on by the DG present. As shown in Fig. 2D, our combined reaction eliminates the plateau during glucose-production with a more than 40-fold enhancement of hydration, compared with the glucose formation at 5 min of Fig. 2C. HPLC analysis of resulting glucose revealed its α-anomeric configuration (Fig. 2E), indicative of *trans*-addition by DG belonging to 13AG (Fig. 1E). It is considered that the plateau (Fig. 2C) was formed by restricted supply of 1,2-enol AF from hydrated AF (Fig. 1C). These findings indicate that α-glucosidase hydrates 1,2-enol AF that has been newly produced by GL from soluble starch, to form the α-glucose, also suggesting the presence of a novel metabolic pathway (Fig. 1D), in which the combined reaction with GL and DG generates glucose from starch in all organisms having GL.

We tried to apply the same combined reaction to 31AG, but all of our 31AGs exhibited the hydrolytic activity on soluble starch. Therefore, we performed a 1-alcohoxy-2-hydro-addition to AF using highly concentrated ethanol-stable ANG (28) (Fig. 1F). The TLC-isolated product from an ANG-mediated reaction on AF and 80% ethanol was determined to be an ethyl α-glucoside by MS and NMR analyses (Fig. 1G). Formation of ethyl α-glucoside indicated that 31AG also catalyzed the *trans*-addition on AF (Fig. 1E).

It is interesting that GlcAL, having no

2-OH-group of AF (Fig. 1B), could not inhibit the HBG2-catalyzed hydrolytic reaction on *p*NPG, even though we used 100 mM GlcAL for this experiment. HBG2 is a 13AG and 13AGs do not have any hydration ability at GlcAL. Therefore, GlcAL does not enable incorporation to the catalytic site of any 13AG. Frandsen *et al*. also suggested that 2-OH is not involved in stabilizing the transition state by GH 13 enzymes (29), one of which is 13AG.

There are two AF-formation systems: i) GL-associated starch degradation and ii) pyranose 2-oxidase (P2O)-catalyzing oxidation at 2-OH-group of glucose, forming a 2-keto AF (Fig. 1C) (30), followed by tautomerization. P2Os, which are found in many fungi, are extracellular (or periplasmic) flavoproteins (30-32), indicating a presence of AF out of cells. In nature, therefore, extracellular α-glucosidases (e.g. 13AGs secreted from bacteria) may convert AF to glucose, which is immediately incorporated into other cells (e.g. 13AGs-secreting bacteria cells). AF, synthesized by extracellular GL, is also altered to glucose by the same mechanism. Recently, Yu (33) found that a fungus *Aspergillus niger* was able to use AF as the sole carbon source for growth, while Bakers' yeast could not grow up in the presence of 0.47 M AF in medium. This observation is of interested, since *A. niger* secrets large amounts of 31AG into medium (9), but α-glucosidase (13AG) of yeast stays inside of cells (34, 35). In nature, microorganisms receive glucose from AF by secreting α-glucosidase, implying the presence of salvage pathway of un-utilized AF.

CONCLUSION

The data indicate that i) α-glucosidases catalyze a *trans*-addition on AF; ii) GlcAL without the 2-OH-group of AF cannot bind to the catalytic site of 13AG, although 31AG can; iii) alkyl α-glucosides are synthesized by α-glucosidase-associated 1-alcohoxy-2-hydro-addition to AF; iv) the combined reaction of GL and α-glucosidase produces glucose from soluble starch. Our study also suggests a novel metabolic pathway of α-glucosidase-associated conversion of AF to glucose in nature, salvaging un-utilized AF.

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FOOTNOTES

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The abbreviations used are: AF, 1,5-anhydro-D-fructose; α2dGlc, α-2-deoxyglucose; CBE, conduritol B epoxide; FD-MS, field desorption mass spectrometry; GH, glycoside hydrolase family; GlcAL, D-glucal; HPAEC, high-performance anion-exchange chromatography; *p*NP, *p*-nitrophenol; *p*NPG, *p*-nitrophenyl α-glucoside.

FIGURE LEGENDS

FIGURE 1. α-Glucosidase-catalyzed reactions (A, B, E, F), conversion of AF under aqueous condition (C), combined reaction of GL and DG (D), and structure of ethyl α-glucoside. (A): 31AG-catalyzed hydrolytic reaction on *p*-nitrophenyl 2-deoxyglucoside to form α-2dGlc and *p*NP (16). (B): 31AG-catalyzed *trans*-addition to GlcAL (17, 18). In water (R=H), 31AG produces α-2dGlc with newly formed axial proton (shown by italic boldfaced H). Alkyl alcohol (R=alkyl) is also used to form alkyl α-2-deoxyglucoside (28). (C): Tautomerization of AF (19-21). (D): GL produces 1,2-enol AF from starch, followed by conversion to α-glucose with DG. (E): α-Glucosidase-catalyzed *trans*-addition on 1,2-enol AF; (F): ANG-catalyzed 1-alcohoxy-2-hydro-addition to 1,2-enol AF.

FIGURE 2. Analysis of reaction products from AF. (A) Representative results of TLC-analysis. 1, without enzyme; 2, ANG; 3, BWG; 4, HBG2; AF, 1,5-anhydrofructose; Glc, glucose; Man, mannose. (B) Representative results of HPAEC-analysis. (B). 1, methyl α-glucoside; 2, glucose; 3, mannose; 4, AF; S, standard sugars; a, without enzyme; b, ANG; c, BWG; d, HBG2. (C) Time-course of DG-catalyzed glucose-formation from AF. (D) Combined reaction of GL and DG on soluble starch (see Fig. 1D). (E) Anomeric configuration of product from Fig. 2-D (5 min-reaction). S, standard sugars including anomerically equilibrated glucose; GL, GL-formed product (1,2-enol AF); GL + DG, combined reaction of GL and DG; α, α-glucose; β, β-glucose.

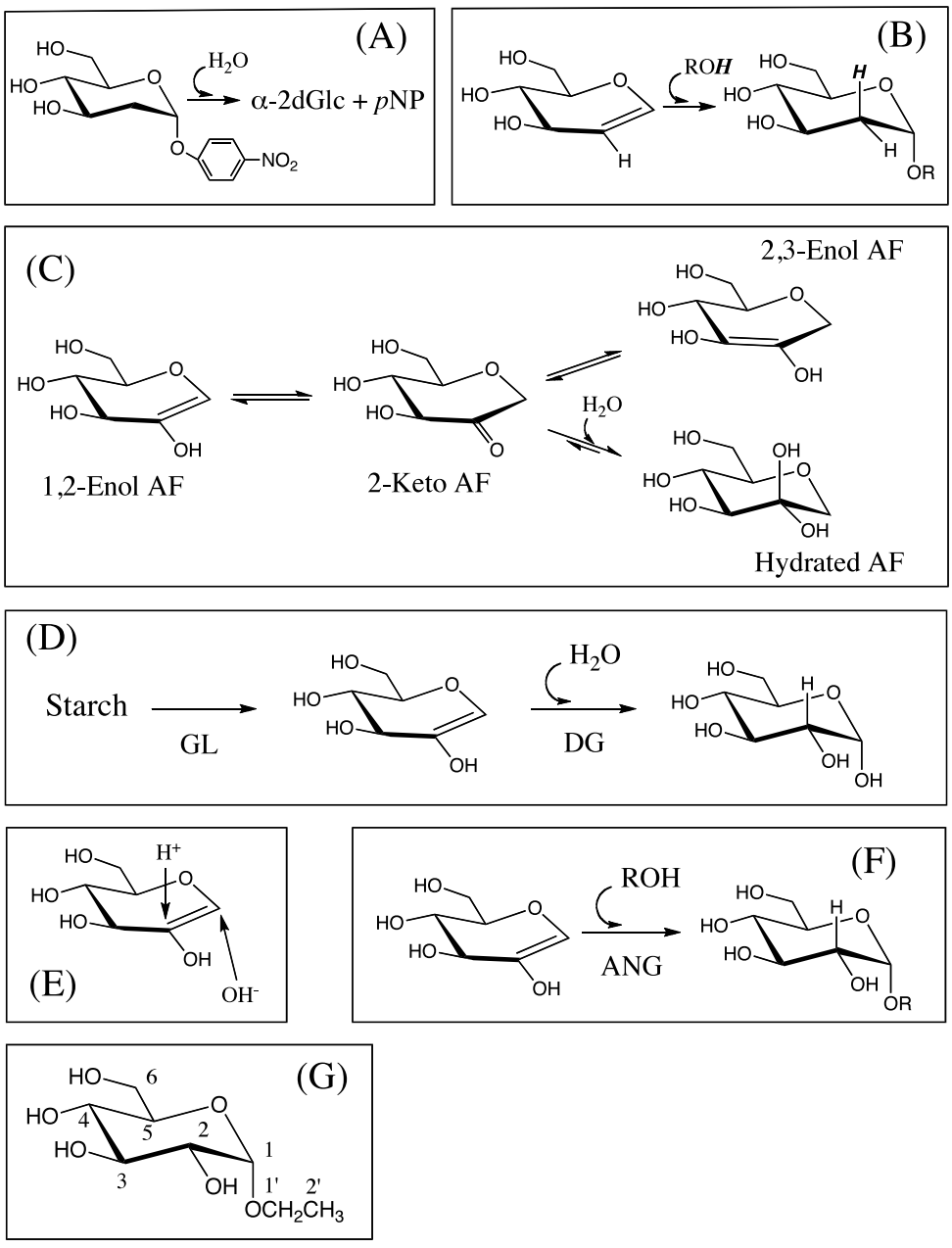


Fig. 1. Kim et al.

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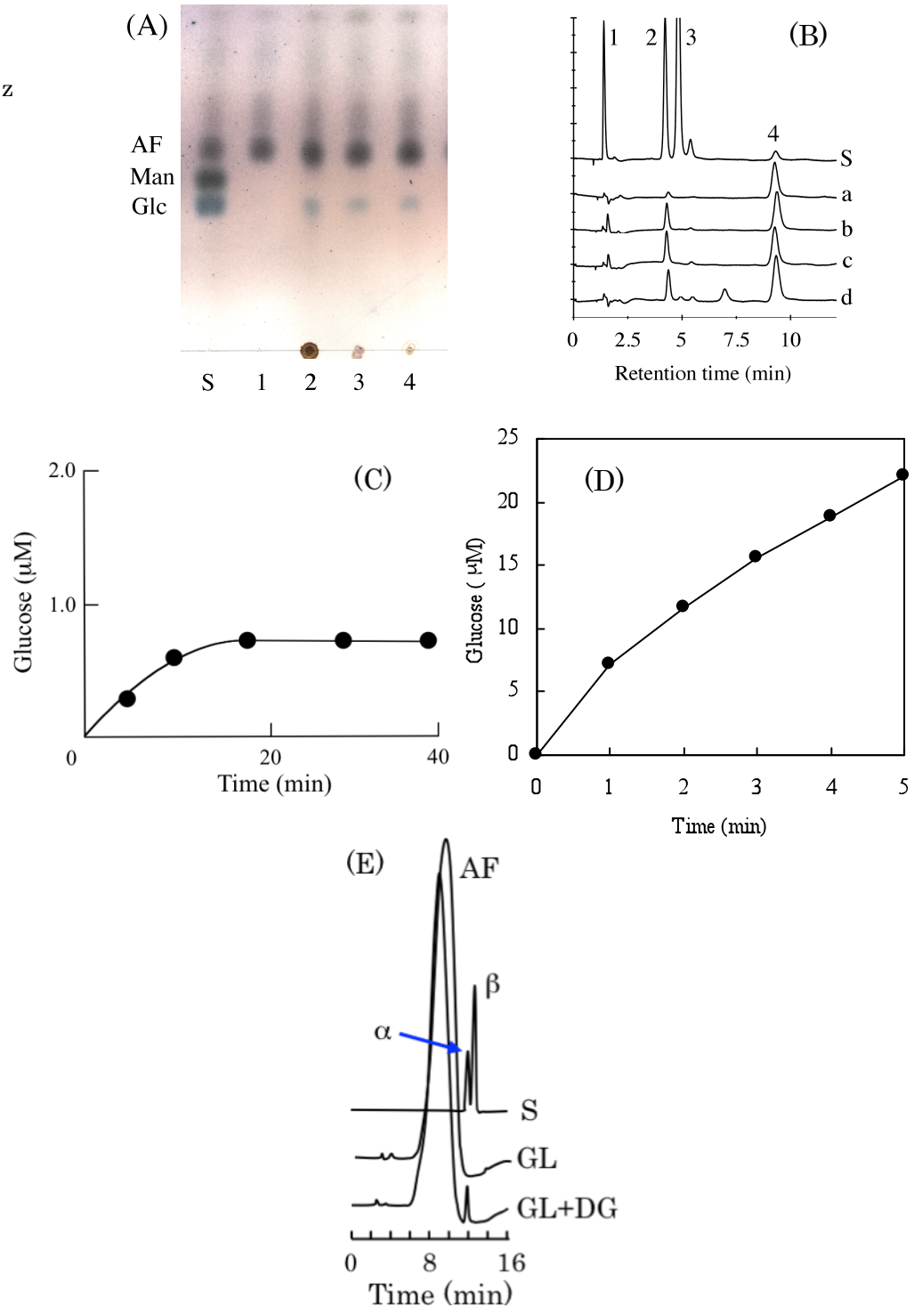


Fig. 2. Kim et al.