There is great interest in using huge amounts of cellulosic biomass, broken down into sugars, as a renewable source. Cellulases have potential applications in various areas, such as the livestock, food, pharmaceutical, textile, detergent, chemical, and fuel production industries. For efficient biological conversion of cellulosic biomass into value-added products or application of cellulose-degrading enzymes, the molecular cloning of cellulolytic enzyme genes with high activity is of the utmost importance. The bioconversion of cellulose into glucose requires the actions of three types of enzymes: a cellulase enzyme complex, including endo-$\beta$-$1,4$-glucanase, cellobiohydrolase, and $\beta$-$glucosidase$ (Coughlan, 1985). Among these, endo-$\beta$-$1,4$-glucanase ($1,4$-$\beta$-$D$-glucan glucanohydrolase; EC 3.2.1.4) attacks cellulose chains at random, breaking internal bonds into smaller fragments, and progressively generating nonreducing ends on which cellobiohydrolase can act. Numerous studies have described endo-$\beta$-$1,4$-glucanase genes in bacteria and fungi (Gilkes et al., 1991). Members of the industrially important $Bacillus$ also produce endo-$\beta$-$1,4$-glucanase, and the genes encoding these enzymes have been cloned and characterized (Lee and Pack, 1988; Baird...
et al., 1990; Lemaire and Beguin, 1993; Miyatake and Imada, 1997). The rumen in ruminants is an especially active site for the fermentation of cellulose. The most abundant cellulolytic ruminal bacteria include *Ruminococcus albus*, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Butyrivibrio fibrisolvens* (Forsberg, 1993; Malburg and Forsberg, 1993; Sahu et al., 2004). The first isolation and identification of a novel anaerobic cellulolytic *Actinomyces* sp. from rumen of Korean native goat was reported by Park (Park et al., 1993). This bacterium having high carboxy methyl cellulase (CMCase; endoglucanase) activity was one of the predominant species in the rumen of Korean native goat and secreted considerable amounts of the enzyme into the culture supernatant (Park et al., 1993; Min et al., 1994).

For potential industrial applications, we had previously conducted extensive screening tests to isolate bacterium with high cellulose activity from rumen of Korean native goat (KNG). After a series of experiments, we reported isolation and identification of cellulolytic *Actinomyces* sp. KNG 40 having the highest cellulolytic activity of isolates tested and characterization of endo-β-1,4-glucanase (Min et al., 1994a,b). Molecular cloning of novel and powerful cellulase genes might be very important for the successful production and industrial application of the enzyme. For this reason, this study was performed to clone novel and powerful cellulase genes from *Actinomyces* sp. KNG 40 and to characterize purified recombinant cellulase enzyme for industrial applications. To the best of our knowledge, complete molecular cloning, sequencing and biochemical characteristics of cellulase from the *Actinomyces* have not been reported yet.

Here, we report, for the first time, the cloning and nucleotide sequences analysis of a novel endo-β-1,4-glucanase gene from *Actinomyces* sp. KNG 40 isolated from the rumen of KNG, and the enzyme properties of an endo-β-1,4-glucanase when expressed in *Escherichia coli* (E. coli) DH5α.

**MATERIALS AND METHODS**

**Bacterial strains and media**

Ruminal fluid was collected from the rumen of KNGs through a cannula to isolate cellulose-degrading bacteria. The collected ruminal fluid was filtered through cheesecloth and diluted with an anaerobic dilution solution using an anaerobic culture system. The colonies were then isolated by the roll-tube method. *Actinomyces* sp. KNG 40 was anaerobically grown at 37°C on Dehority’s artificial medium containing 0.2% cellobiose. *E. coli* DH5α was grown aerobically in Luria-Bertani (LB) medium at 37°C or on an LB plate, supplemented with ampicillin (50 µg/mL) for transformants.

**Construction of a genomic DNA library and cloning of endoglucanase**

The pUC19 plasmid and *E. coli* DH5α were used as the vector-host system for cloning. Chromosomal DNA of *Actinomyces* sp. KNG 40 was prepared from the cells in the early exponential growth phase following the method of Saito and Miura (1963). Plasmid DNA was prepared by the alkalai lysis method (Sambrook et al., 1989). Competent cells of *E. coli* were prepared by the calcium chloride method (Sambrook et al., 1989). Chromosomal DNA of *Actinomyces* sp. KNG 40 was partially digested with *Sau3AI* and separated by sucrose density gradient centrifugation (10% to 40% w/v). DNA fragments of 2 to 8 kb were collected and ligated into the *BamH* I site of the pUC19 vector. The ligation products were transformed into competent *E. coli* DH5α, and transformants were selected for by plating on LB agar plates, supplemented with X-gal (5-bromo-4-chloro-3-indolyl-galactoside) and ampicillin at 37°C for 12 to 14 h. Clones expressing endoglucanase activity were detected by replica plating the bacterial colonies onto LB containing 0.5% carboxymethyl cellulose (CMC) and ampicillin. The plates were incubated at 37°C for 16 to 24 h. The plates were then flooded with a 0.1% aqueous solution of Congo red (Sigma, St. Louis, MO, USA) (Teather and Wood, 1982) for 30 min. Unbound dye molecules were removed by reflooding the plates with 1 M NaCl for 15 min. The formation of visible halos around the colonies indicated the production of endoglucanase.

**DNA sequence analysis**

DNA sequences were determined by the chain termination method of Sanger et al. (1977) using a PRISM-ready reaction dideoxy terminator cycle sequencing kit (Perkin Elmer, Boston, MA, USA) with supercoiled plasmids as templates. The DNA sequences were analyzed using the PC/GENE software package (V. 6.60, IntelliGenetics Inc., Mountain View, CA, USA).

**Purification and N-terminal amino acid sequence analysis of an endoglucanase produced by *E. coli* DH5α**

An endoglucanase produced by *E. coli* DH5α containing pDS3 was purified by a crystalline cellulose (Avicel PH-101; Fluka Chemie GmbH, Buchs, Switzerland) column. The cultured cells were harvested at an early stationary phase by centrifugation at 3,400× g for 10 min, and the supernatant was used as a crude enzyme source. The enzyme solution was concentrated using ultrafiltration on an Amicon YM-10 membrane (MW cut-off: 10,000) in an Amicon model 8400 (Millipore, Bedford, MA, USA) stirring ultrafiltration cell under 20 psi of N2. The solution was concentrated by ultrafiltration at each purification step, if necessary. The concentrated enzyme was placed in a crystalline cellulose column (20 mL bed volume) using a BioLogic System (Bio-Rad, Hercules, CA, USA). Bound
proteins were eluted with double-distilled water and then buffer exchanged into 20 mM Tris-Cl, pH 7.4. The purified endoglucanase (3.0 μg/well) was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis minigel at 200 V using a Bio-Rad Mini-PROTEAN cell. Proteins were electroblotted at 70 V for 1 h with water cooling onto a Trans-Blot polyvinylidene fluoride membrane (Bio-RAD Laboratories, USA). The membrane was immersed in 0.025% Coomassie Blue R-250 for 5 min and destained with 50% methanol for 15 min. The membrane was then washed with a large volume of distilled water with several changes. The protein band was cut out, and the N-terminal amino acid of the protein was microsequenced using an Edman Gas Phase Microsequencer (Model ALI 473a; Applied Biosystems Inc., Foster city, CA, USA).

**RESULTS AND DISCUSSION**

**Gene cloning of an endoglucanase from *Actinomyces* sp. Korean native goat 40**

The genomic library was constructed by ligating 2 to 8 kb Sau3AI fragments of *Actinomyces* sp. KNG 40 DNA into the *BamH*I site of pUC19. To isolate endoglucanase genes, clones expressing endoglucanase activities were detected by replica plating the bacterial colonies onto an LB (with ampicillin) plate containing 0.5% CMC. Among the 1,250 clones screened, 10 were identified as endoglucanase positive based on the formation of visible halos around the colonies. Only one of the endoglucanase-positive clones was identified and confirmed as having assayable endoglucanase activities. A recombinant plasmid harboring a 5.0 kb insert from this clone was designated as pDS1. Various restriction fragments from pDS1 were subcloned into pUC19 vectors, and endoglucanase activity was determined on the CMC plate. *E. coli* clones containing pDS3 (3.2 kb insert) showed the highest endoglucanase activity (Figure 1). It has been shown conclusively that most cellulolytic microorganisms produces the isomeric forms of cellulase and xylanase with similar substrate specificities, which are the products of large multigene families but not the result of post-translational modification of a single enzyme (Malburg and Forsberg, 1993; Bedford and Partridge, 2001; Nguyen et al., 2012, Rashamuse et al., 2013). Therefore, this clone might be one of multiple cellulase genes from *Actinomyces* sp. KNG 40.
and sequenced, and their amino acid sequences have been deduced (Pollle et al., 1990; Mittendorf et al., 1993; Cho et al., 2000; Nguyen et al., 2012). However, to our knowledge, molecular cloning, sequence analysis and characterization of cellulase gene from anaerobic rumen bacterium Actinomyces sp., has not been reported so far. This is the first report of the cloning and sequence analysis of an endoglucanase gene from rumen bacterium Actinomyces sp. No DNA sequence similarity was identified with the published endoglucanase from a variety of bacteria. The GenBank accession number of the cloned endoglucanase is U94825.

Purification and N-terminal sequence analysis of endoglucanase produced by E. coli DH5α

Fungal cellulosases are usually released from the cell (Areej et al., 2014). Although generally bacterial enzymes seem to remain with cell, cellulosases from some anaerobic rumen bacteria may be secreted by the cell (Clarke, 1997). For examples, endoglucanases from some rumen bacteria including Clostridium sp., Fibrobacter succinogenes, and Bacillus licheniformis were reported to be secreted into the culture medium (Mittendorf and Thomson, 1993; Cho et al., 2000; Seo et al., 2013). In this study, two-thirds of the endoglucanase enzyme was secreted into the Actinomyces culture medium. About 40% of the recombinant enzyme was fully secreted into the culture medium when it was expressed in E. coli containing pDS3 (Table 2). It means that the secretion ability of the signal peptide of the endoglucanase is more powerful than others, and the secreted recombinant enzyme in E.coli can be used in many industrial applications including animal feed industry (Kuhad et al., 2011; Gao et al., 2015). A recombinant endoglucanase EG1 was purified from the culture medium of E. coli DH5α containing pDS3 in one step in a microcrystalline cellulose (Avicel PH-101; Fluka Chemie GmbH, Buchs, Switzerland) column. The specific activity of the purified EG1 was 56.7 U/mg protein.

The SDS-PAGE/zymogram technique using the fluorogenic substrate methylumbelliferyl cellobioside (MUC) was used to separate and detect two major bands showing endoglucanase activity from the purified enzyme. An active protein bands having an approximate molecular weight (MW) of 51.4 and 57.1 kDa, respectively, were detected on Laemmli gel under UV illumination (data not shown) (Figure 2). The ratio of the activity and the protein band intensity of the two bands were very similar. The N-terminal sequences of these two bands analyzed by Edman degradation method matched those of the deduced sequences, starting from residue 166 and 208, respectively (Figure 3). The cleavage site of the recombinant endoglucanase by signal peptidase obey the

Table 1. Comparison of the amino acid sequences of the endoglucanase in this study with those various family 5 endoglucanases

| Protein             | Species                        | Family | Identity (%) | Positivity (%) | GenBank No. |
|---------------------|--------------------------------|--------|--------------|----------------|-------------|
| Endoglucanase       | Fibrobacter succinogenes       | 5      | 67           | 80             | U94826      |
|                     | Clostridium saccharobutylicum   | 5      | 43           | 59             | P15704      |
|                     | Bacillus subtilis DLG           | 5      | 40           | 57             | A26874      |
|                     | Bacillus amyloquefaciens       | 5      | 41           | 58             | AF363635    |
|                     | Ruminococcus albus             | 5      | 42           | 61             | AB016777    |
|                     | Clostridium acetobutylicum      | 5      | 37           | 55             | AE007585    |

1 The comparison of the amino acid sequences was performed with the NCBI’s BLAST program.

Table 2. Localization of endo-β-1,4-glucanase activity (in %) in Actinomyces sp. Korean native goat 40 and Escherichia coli (E. coli) DH5α

| Host             | Extracellular | Periplasmic | Cytoplasmic | Cell-bound | Total activity |
|------------------|---------------|-------------|-------------|------------|----------------|
| Actinomyces      | 67.7          | 11.3        | 10.5        | 10.5       | 100            |
| E. coli          | 39.8          | 18.7        | 31.8        | 9.6        | 100            |
“(–3, –1) rule”: the residue at position -1 must be small and uncharged (Ala, Ser, Gly, Cys, Thr, Gln, Pro, or Leu), and also the residue at position -3 is always a small and uncharged (Ala, Ser, Gly, Cys, Thr, Ile, Leu, or Val) (Perlman and Halvorson, 1983; Von Heijne, 1985; Yan and Wu, 2014). It indicates that these preceding amino acids play a signal peptide-like role. The cellulose-binding domain starts from residue 166 or 208.

Characterization of purified endoglucanase produced by *E. coli* DH5α

Table 3 shows the substrate specificity of endoglucanase. Many studies have reported that a large number of cloned cellulosomes from rumen bacteria possess bifunctional endoglucanase-xylanase activities (Cho et al., 2000; Chang et al., 2011; Rashamuse et al., 2013; Yuan et al., 2015). The purified endoglucanase from our study showed the highest enzyme activity against CMC but weak enzyme activity was observed for xylan, avicell, and cellobiose (Table 3).

**Table 3. Substrate specificity of endoglucanase from *Escherichia coli* DH5α harboring pDS3**

| Substrate            | Relative activity (%) |
|----------------------|-----------------------|
| Carboxymethyl cellulose¹ | 100                   |
| Xylan¹               | 6.1                   |
| Avicell¹             | 8.1                   |
| Cellobiose²          | 26                    |

¹ Enzyme activity was measured as the amount of reducing sugars released.
² Enzyme activity was calculated from the amount of glucose released and divided by two.
This result is consistent with previous studies of *Actinomyces* sp. (Min et al., 1994).

The optimum temperature of the enzyme activity was determined by assaying the endoglucanase activities at various temperatures for 30 min in a 50 mM sodium citrate buffer (pH 6.0) with 0.7% CMC. The optimal temperature of the purified enzyme was 55°C, but it retained over 90% of maximum activity in a broad temperature range (40°C to 60°C). The enzyme activity decreased rapidly above 65°C but the enzyme still retained 60% of its maximum activity even at 70°C (Figure 4). In general, endoglucanases from fungi and bacteria are stable at temperature below 55°C (Ohara et al., 2000; Nguyen et al., 2012; Culleton et al., 2014). This result suggests that the cloned endoglucanase from *Actinomyces* sp. KNG 40 is more thermostable and active at high temperature.

The effects of pH on the activity were determined by varying the pH of the reaction mixture using a 50 mM sodium citrate buffer (pH 3.0 to 6.0) and 50 mM sodium phosphate buffer (pH 6.0 to 8.0) with 0.7% CMC at 45°C for 30 min. The optimal pH for the enzyme activity was 6.0 (Figure 5). This result is consistent with previous reports. Many studies have reported that the optimal pH for the cloned endoglucanases activity from various cellulolytic rumen bacteria was in the range of pH 5.0 to 7.0 (Cho et al., 2000; Gong et al., 2012; Nguyen et al., 2012). The enzyme kinetic parameters were measured in a 50 mM sodium citrate buffer (pH 6.0) at 55°C for 30 min. From linear plots of the Michaelis-Menten equation such as Lineweaver-Burk or Hanes-Woolf plot using an Excel curve fitting program, the $K_m$ and $V_{max}$ of recombinant EG1 was calculated: In Lineweaver-Burk plot ($R^2 = 0.9998$) $K_m$ and $V_{max}$ were 0.4% CMC and 144.9 U/mg, respectively. In Hanes-Woolf plot ($R^2 = 0.9997$) they were 0.39% CMC and 143 U/mg, respectively.

In conclusion, we have successfully cloned a novel endo-β-1,4-glucanase gene from cellulolytic rumen *Actinomyces* sp. KNG 40 bacterium into *Excherichia coli*, and analyzed nucleotide sequences and the enzymatic properties of the cloned endoglucanase. The cloned endoglucanase is more thermostable than enzymes produced by other rumen bacteria, and has powerful signal sequences for secretion of the enzyme into culture medium. Therefore, this recombinant endoglucanase may be a good candidate to be used in livestock feed as an additive to increase the digestibility of feedstuff for non-ruminant animal, and in the other industry.

**CONFLICT OF INTEREST**

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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