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ORIGINAL PAPER

# Identification of three proteins up-regulated by raw starch in *Cytophaga* sp.

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**Abstract** Raw starch-digesting amylases (RSDAs) in many microorganisms convert starch granules into maltodextrins and simple sugars. We cloned and sequenced from *Cytophaga* sp. an RSDA with an excellent raw starch digestion activity. This RSDA was highly inducible by raw starch, but not by other sugars, suggesting that an unknown signal transduction mechanism is involved in the degradation of raw starch. We used a proteomic approach to investigate the effect of raw starch on protein expression in *Cytophaga* sp. Using MALDI–TOF MS protein analysis, we have identified three proteins up-regulated by raw starch, i.e., a 60-kDa chaperonin (cpn60), glutaminase, and pyruvate phosphate dikinase (PPDK). Subsequent time-course studies detected an increased expression of RSDA as well as the highest expression of PPDK occurring 6 h post-incubation with raw corn starch, implying that the latter enzyme may work along with RSDA on the digestion of raw starch. Finding these proteins up-regulated by raw starch may provide an insight into how *Cytophaga* sp. cells respond to raw starch stimulation.

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**Keywords** *Cytophaga* sp. · Raw starch · Two dimensional-gel electrophoresis

**Abbreviations**

| | |
|---|---|
| CBB | Coomassie Brilliant Blue R250 |
| cpn60 | 60-kDa chaperonin |
| GWD | α-Glucan, water dikinase |
| IEF | Isoelectric focusing |
| MALDI | Matrix-assisted laser desorption ionization |
| PPDK | Pyruvate, phosphate dikinase |
| RSDA | Raw-starch-digesting amylase |
| 2-DE | Two dimensional-gel electrophoresis |

## Introduction

Starch, the major carbohydrate source in the world, is synthesized and stored in granulated form in plants. Starch granules are semi-crystalline particles composed of amyloses, which are linear chains of α-1, 4-linkaged glucose units, and high branched amylopectin, which are polysaccharides with an α-1, 4-backbone and α-1, 6-branched points (Blennow et al. 2002). Many starch digesting enzymes have been identified to date. For example, α-amylases (1,4-α-D-glucan glycanohydrolase, EC3.2.1.1) randomly cleave α-1,4-glucosidic bonds in starch, forming oligosaccharides; β-amylases (EC 3.2.1.2) and glucoamylases (EC 3.2.1.3) hydrolyze starch from its non-reducing end and produce maltose and glucose (Pandey et al. 2000). These enzymes are widely used in the food industry for starch processing (Biwer et al. 2002; van der Maarel et al. 2002).

In order to improve the conversion of starch molecules into simple sugars by amylolytic enzymes, starch granules have to be gelatinized in water. However, this gelatinization

process requires heating and a long reaction time (Colonna et al. 1992). Raw starch-digesting amylases (RSDAs), which digest raw starch directly, therefore, have been used to reduce the cost and increase the efficiency of starch processing (Nielsen and Borchert 2000). In previous studies, we demonstrated that a Gram-negative bacterium *Cytophaga* sp., produces a raw-starch-digesting-amylase that displays an excellent raw-starch-digestion activity (Jeang et al. 1995). Interestingly, this RSDA is only highly expressed under raw starch stimulation, but not in response to 14 other carbon sources like soluble starch, fructose, galactose, and dextrose (Chiou and Jeang 1995). This suggests that raw starch metabolism may involve an unknown signal transduction mechanism. To investigate this possibility, we have compared the protein expression patterns of *Cytophaga* sp. cells cultured in the presence and absence of raw corn starch. Our results showed that the expression levels of 15 proteins were elevated by raw starch stimulation. Among these raw starch-induced proteins, three were identified as 60 kDa chaperonin, glutaminase, and pyruvate phosphate dikinase, by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF MS) analysis.

## Materials and methods

### *Cytophaga* sp. cells culture

*Cytophaga* sp. cells from a stock kept at −20°C were streaked on an NS plate (2.3% nutrient agar and 1% soluble starch), and incubated at 37°C overnight. A single colony was taken from the plate, streaked again on a fresh plate, and allowed to incubate at 37°C overnight. A single colony from the second NS plate was then inoculated in a 5 mL YPs medium (0.2% Yeast extract and 0.5% Polypeptone-S) at 37°C (100 rpm) for 12 h. After incubation, cells were diluted to 100-fold with the same medium in the presence or absence of 0.2% raw corn starch (dry heat sterilization at 105°C for 2 h) and cultured in a 500 mL Erlenmeyer Hinton flasks at 37°C (100 rpm). At time intervals, cells were transferred and centrifuged at 8,000 rpm for 10 min. The supernatants and cell pellets were collected for further assays.

### Sample preparation and Western blot assay

To precipitate proteins, 1 mL supernatant of each sample was mixed with 0.1 mL of trichloroacetic acid (TCA) and placed at −20°C for 30 min. The tubes were centrifuged (12,000 rpm) at 4°C for 10 min. The pellets were washed twice with 1 mL of 70% ethanol, vacuum dried and resuspended in 40 μL of Tris–HCl buffer (10 mM, pH 8.0). For Western blot assay, 10 μL of fivefold SDS-PAGE sample buffer was added into each tube, and then duplicate 20 μL samples from each tube were loaded onto two 10% acrylamine gels. After electrophoresis, proteins on one gel were transferred to a nitrocellulose membrane, and the other gel was stained with 0.3% Coomassie Brilliant Blue R250 (CBB). The membrane was incubated with a TBS blocking buffer (20 mM Tris–HCl, 150 mM NaCl and 5% skim milk, pH 7.4) for 1 h. After incubation, the membrane was soaked in a 5,000-fold diluted anti-RSDA antibody solution for 2 h, washed three times with TBST buffer (TBS buffer containing 0.05% Tween-20) for 5 min, incubated in a TBS blocking buffer containing a 2,000-fold dilution of a peroxidase conjugated secondary antibody solution (Goat anti-rabbit-IgG), and soaked for 2 h in the dark. The membrane was washed three times with TBST buffer and incubated with substrate solution (2.5 mL of methanol containing 2.5 mg of 4-chloro-1-naphthol, 12.5 mL TBS buffer, and 50 μL of 30% $H_2O_2$). Once the color bands appeared, the reaction was quenched by washing the membrane with distilled water and air drying.

### Extraction of membrane proteins from *Cytophaga* sp. cells

*Cytophaga* sp. cells were taken 4, 6, and 8 h post-inoculation with or without raw starch, filtered twice, and then centrifuged at 8,000 rpm for 20 min. The cell pellets were washed with ST buffer (250 mM sucrose, 10 mM Tris–HCl buffer, pH 7.0), and then resuspended in a 10 mL of Tris–HCl buffer (10 mM, pH 8.0). The cells were lysated by a FRENCH® pressure cell, followed by centrifugation at 12,000 rpm for 10 min at 4°C to precipitate the nuclear acids and cell debris. The supernatants were transferred to 5 mL ultracentrifugation tubes and centrifuged at 60,000 rpm for 45 min at 4°C by micro ultracentrifuge CS150GXL (S100AT rotor, Hitachi Koki Co. Ltd, Ibaraki, Japan). The pellets (envelopes) were resuspended in a 1 mL Tris–HCl buffer (10 mM, pH 8.0) containing 30% sterilized glycerol, aliquoted, and stored at −70°C for further assays.

### Two dimensional electrophoresis (2-DE) analyses

Approximate 1 mg of cell envelope proteins of the control cells and 1.5 mg membrane proteins of corn starch-treated cells were used for IEF assays on an Ettan™ IPGphor Isoelectic Focusing System (Amersham Bioscience, Uppsala, Sweden). The samples were diluted with rehydration buffer (8 M Urea, 2% Triton X-100, 18.2 mM DTT, 0.5% IPG buffer pH 3–10 and 0.002% Bromophenol blue) to a final volume of 250 μL and loaded into a ceramic strip holder. An immobiline DryStrip (13 cm, pH 4–7) was put into the holder and covered with IPG cover fluid, and the holder was transferred to an IPGphor platform. The electrophoresis

was performed according to the manufacture's instructions. Briefly, the DryStrip gels were soaked in a 10 mL SDS-equilibration buffer (50 mM Tris–HCl pH 8.8, 6 M Urea, 30% Glycerol, 2% SDS, and 0.002% Bromophenol blue) containing 100 mg of DTT for 15 min. The gels were then incubated in a 10 mL SDS-equilibration buffer containing 250 mg of idoacetamide for 15 min, washed with an electrophoresis buffer and fixed on the top of a 12.5% SDS-PAGE gel with 1% agarose solution. The SDS-PAGE analysis was run on a HoferTM SE 600 Ruby vertical electrophoresis unit (Amersham Bioscience) with PAC3000 power supply (BIO-RAD Laboratories Inc., CA, USA). To the gels were applied 15 mA for 15 min, followed by 60 mA until the bromophenol blue ran out of the gel. After running, the gels were stained with CBB.

### Protein identification

The 2-D gels were scanned by ImageScanner (ImageMaster™ Labscan, Version 3.01b, Amersham Bioscience), and the relative protein concentration was quantified using the 2-DE analysis software ImageMaster™ 2D Elite Software, Version 2002.02 (Amersham Bioscience). Interested proteins were isolated from gels and subjected to MALDI–TOF MS analysis (Taiwan Proteins Corp.).

## Results and discussion

### RSDA is an extracellular protein

The location of RSDA in *Cytophaga* sp. was determined by Western blot assay using antibodies against RSDA. *Cytophaga* sp. cells were cultured with or without 0.2% raw corn starch in a YPs medium. Cell cultures were centrifuged to separate the cell pellets and supernatant. Proteins in supernatant fractions were precipitated by TCA. As shown in Fig. 1, under raw starch stimulation, RSDA was observed in the protein sample precipitated from the culture medium. No detectable RSDA was found in cell pellets.

To determine when the RSDA was induced by raw starch, a time-course study was performed. As shown in Fig. 2, RSDA was detected at 6 h after inoculation in the presence of corn starch. No detectable RSDA was found in samples taken from cells cultured in the absence of this raw starch.

### 2-DE analysis of protein regulated by raw corn starch

To investigate whether raw starch stimulation also affects the expression of other cellular proteins, membrane proteins from *Cytophaga* sp. cells cultured with or without 0.2% raw corn starch for 6 h were separated by 2-DE and visualized by CBB staining. ImageMaster™ 2D Elite Software was used to measure the intensity of protein spots between the control and raw starch-treated samples. As shown in Fig. 3, 27 proteins consistently showed changes in their levels under raw starch stimulation. To further characterize these raw starch-regulated proteins, a time course study was performed. Samples from cells cultured with or

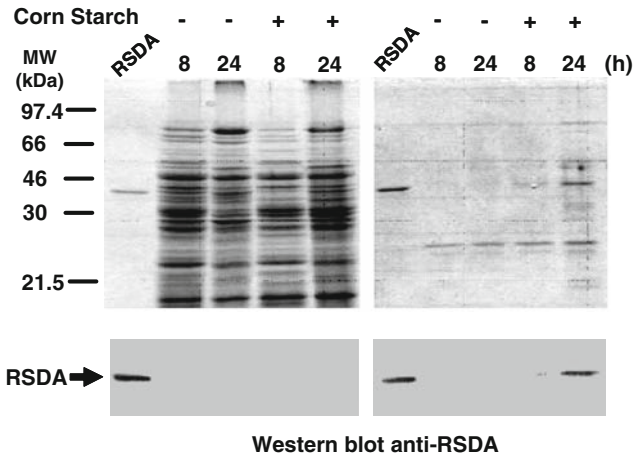


**Fig. 1** Cellular location of RSDA in *Cytophaga* sp. total cell lysate (*left panel*) and supernatant (*right panel*) were subjected to SDS-PAGE. The proteins were stained with CBB (**a**) and RSDA was detected by Western blotting (**b**)

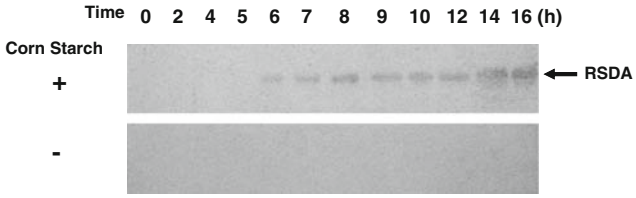


**Fig. 2** RSDA expression profile in response to raw starch incubation. *Cytophaga* sp. cells were cultured in YPs medium in the presence (*upper*) or absence (*lower*) of 0.2% corn starch for the indicated times. Proteins were precipitated from supernatant fractions and detected by Western blotting

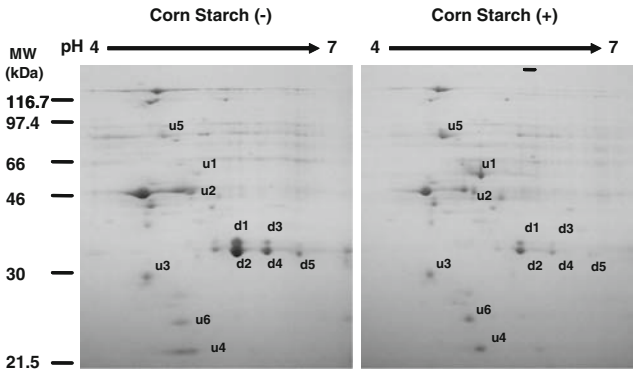


**Fig. 3** Analysis by 2-DE of membrane proteins from *Cytophaga* sp. *Cytophaga* sp. cells were cultured in YPs medium or YPs medium containing 0.2% corn starch for 6 h. Membrane proteins were isolated and separated by 2-DE, and visualized by CBB staining

without raw starch, were prepared 4, 6 and 8 h post-incubation. The relative protein concentrations were measured and normalized using samples taken at 4 h as references. Proteins that were increased or decreased at least twofold in each experiment were considered as starch up-regulated proteins (designated u1 to u6) or down-regulated proteins (designated d1 to d5).

The expressions of spots u1 and u6 kept increasing after raw starch was added into the medium, with the maximum expressions of these two proteins occurring 8 h post-incubation. For spots u2 and u4, the expression levels reached maximum as early as 4 h post-incubation with raw starch, and subsequently decreased with further incubation. Significant increase of the expression of spot u3 (fivefold) was observed only after 8 h of incubation with raw starch. The maximum expression of spot u5 occurred 6 h post-incubation with raw starch, and then started to fall. On the other hand, the expressions of five proteins were repressed after incubation with raw starch. Of these, spot d5 only retained 10% of its expression after incubation with raw starch for 8 h (Fig. 4b).

## The possible roles of raw starch up-regulated proteins in starch degradation

Our best interest is to find the proteins which can increase RSDA activity in starch processing. The results given above indicate that a significant induction of RSDA occurred 6 h after the incubation with raw starch (Fig. 2). Therefore, we decided to focus on the proteins which were also highly induced (more than fivefold) 6 h post-incubation. The results of LC/MS analysis showed that spot u1, spot u3, and spot u5 were closely related to 60 kDa chaperonin (cpn60), glutaminase (EC 3. 5. 1. 2), and pyruvate phosphate dikinase (PPDK; EC 2. 7. 9. 1) (Table 1).

60 kDa chaperonin (cpn60) is a member of the chaperone superfamily, which is highly induced in response to heat shock stimulation. The main function of cpn60 is to assist the protein folding and transportation (Saibil 2000). It also has been shown that the expression of cpn60 could reduce insoluble aggregation of recombinant proteins overexpressed in bacteria (de Marco et al. 2005). Since RSDA is highly induced under raw starch stimulation, increasing expression of cpn60 observed in this study might assist the folding and secretion of raw starch-inducible proteins, including RSDA. Further study on the regulation of RSDA by cpn60 is currently underway in our laboratory.

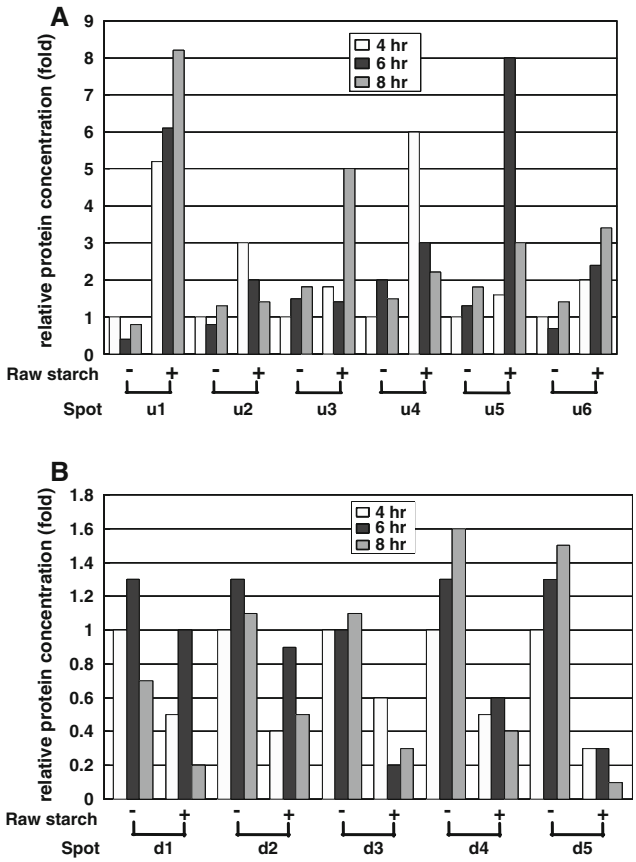


**Fig. 4** Quantification of raw starch-regulated membrane-associated proteins. The relative amounts of corn starch induced (**a**) and repressed (**b**) proteins were measured by ImageMasterTM Labscan, and normalized using samples taken at 4 h as references. Three different incubation times, 4, 6 and 8 h were indicated by white, black or gray colors, respectively

The glutaminases catalyze the conversion of glutamine into glutamate, ammonia, and NADH. In *E. coli*, the expression of glutaminase could be induced by glucose and excess ammonia (Prusiner 1975; Prusiner et al. 1976). In

**Table 1** Identification of 3 raw-corn starch up-regulated protein spots

| Spot | Mr/p*I* (E)[a] | PM[b] | (%C)[c] | Protein name | Species | Accession number | Mr/p*I* (T)[a] |
|---|---|---|---|---|---|---|---|
| u1 | 60/5.5 | 5 | 12 | 60 kDa chaperonin 2 (cell wall protein A) | *Mycobacterium tuberculosis* | P06806 | 56.6/4.9 |
| u3 | 32/5.0 | 5 | 36 | Probable glutaminase | *Streptomyces coelicolor* | P57755 | 32.6/5.4 |
| u5 | 97/5.1 | 6 | 13 | Pyruvate, phosphate dikinase (PPDK) | *Rickettsia conorii* | Q92HI8 | 98.3/6.2 |

[a] Mr/pI(E) or Mr/pI(T): experimental (E) or theoretical (T) relative molecular mass (kDa)/isoelectric point

[b] PM: number of peptides matched

[c] (% C) sequence coverage

this report, we found that the expression of glutaminase in *Cytophaga* sp. was induced 8 h post-incubation with raw starch. Since RSDA was induced 6 h after incubation, induction of glutaminases might be due to increasing glucose levels through the digestion of starch by RSDA.

Pyruvate, phosphate dikinase (PPDK), also named orthophosphate dikinase, is an inorganic pyrophosphate-dependent enzyme (Wood et al. 1977). In the glycolytic pathway, PPDK transfers phosphate groups on phosphoenopyruvate into pyruvate, and produces inorganic phosphate and ATP, one of the energy sources for microorganisms and plants (Evans and Wood 1971; Pocalyko et al. 1990; Eisaki et al. 1999). Recently, α-glucan, water dikinase (GWD), also known as starch-related R1 protein, was cloned and sequenced in plants. GWD contains a starch binding domain and a kinase domain which is similar to the kinase domain found in PPDK (Mikkelsen et al. 2004; Mikkelsen and Blennow 2005). It has been shown that GWD could transfer phosphate groups from ATP onto starch and results in an increasing degradation rate for starch in vitro (Edner et al. 2007). Moreover, in GWD knockout *Arabidopsis* (*Arabidopsis thaliana*), starches were found to have accumulated in leaves (Yu et al. 2001; Ritte et al. 2002). Since no bacterial kinases facilitating the starch processing has been identified yet, it would be worthwhile to know whether PPDK in *Cytophaga* sp. could assist the degradation of raw starch by RSDA. Since PPDK dose not have any known starch binding domain, it is possible that the raw starch up-regulated protein identified herein is a PPDK-like protein with a starch binding domain.

In conclusion, while simple sugar metabolism and the effects of these simple sugars on cellular events have been well studied to date, it is still unclear how bacteria respond to polysaccharide stimulation. Data presented in this report demonstrate that in *Cytophaga* sp., an RSDA, the key player in the hydrolysis of raw starch, is highly induced by the latter. Besides RSDA, three other proteins, i.e., cpn60, glutaminase, and PPDK, were also identified as raw starch up-regulated proteins. Identification of these raw starch regulated proteins has provided a useful tool to study the raw starch metabolism pathway as well as how bacteria respond to raw starch stimulation.

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