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COLLAGENASE AND TRIS-HCL PRE-TREATMENT REMOVE INHIBITORY PROPERTIES OF PORCINE COLLAGEN DURING RT-PCR DETECTION

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ABSTRACT

Collagens have a critical role in the food, cosmetic, and pharmaceutical industries. Collagen comes from a variety of sources, making it crucial for halal considerations. Thus, the possible adulteration and contamination of non-halal collagen should be examined with lab testing. Currently, real-time PCR (RT-PCR) serves as a sensitive and specific method for halal examination. Unfortunately, collagen has inhibitory properties that inhibit amplification in real-time PCR. This study will examine the use of a pre-treatment method to eliminate the inhibitory properties and allow collagen analysis using RT-PCR. In this study, pre-treatment was carried out by hydrolysis using enzymes and acids. The pre-treatment by collagenase and Tris-HCl hydrolysis allowed for porcine DNA in collagen to be detected with a Ct value of 36.15.

Keywords: Bovine collagen, Collagenase enzymes, Hydrolysis, Porcine Collagen, RT-PCR

INTRODUCTION

Collagen can be extracted from the skin and bones of some vertebrate animals, such as bovines and pigs. Therefore, the halal status of collagen depends mostly on the source of raw material used and the production process. Porcine-based collagens are prohibited (haram) for Muslims, while other livestock such as cows can be consumed as long as halal-slaughter procedures are followed. It is recommended that Muslims should refrain from using collagen that has no known source.

Collagen is a unique protein because it has a triple helix structure that distinguishes collage from other proteins in the human body. Collagen has inhibitory properties that inhibit the

amplification of DNA in real-time PCR. Real-time polymerase chain reaction (RT-PCR) is a sensitive and specific method specifically used for halal examination by detecting the source of a material based on the amino acid sequence. Collagen protein cannot be detected directly using RT-PCR, whereas gelatin, which is formed when collagen is partially hydrolyzed, may be detected using RT-PCR. The Ct value of 37.87 for porcine gelatin and 37.48 for bovine gelatin, indicates that gelatin is more sensitive than collagen protein (Cai et al., 2012).

This study aims to determine the efficacy of pre-treatment methods with enzyme and acid hydrolysis so that the collagen hydrolysate can be analyzed using RT-PCR. In general, this research was carried out in several steps, beginning with collagen hydrolysis using enzymes and acids, followed by identification of collagen hydrolysate DNA using RT-PCR. Pig and bovine collagen were obtained from the bones of an animal, collagenase type I from *Clostridium histolyticum* (BioFroxx China), Tris-HCl (Merck KGaA, Darmstadt Germany), papain enzyme (PT. Bromelain Enzyme, Lampung, Indonesia), formic acid (Merck), and acetic acid. Sample extraction was carried out using DNA Extraction Kit from KogeneBiotech Korea, while the primer-probe design usesTaqpath^{TM} ProAmp^{TM} Master Mix combined with IPC TaqMan^{TM} (AppliedBiosystems), identification of collagen DNA using RT-PCR by CFX96 machine (BioRad) (Tanabe et al., 2007).

Collagen inhibits amplification in real-time PCR, therefore pre-treatment to remove the inhibitors is required. This study used enzymes and acids to hydrolyze as the two forms of pre-treatment. First, hydrolysis using 2% formic acid and papain (200 IU). 1 g of collagen was dispersed with 100 mL of 2% formic acid, then heated at 108 ^{0}C for 2 hours, after that added papain at 60 ^{0}C for 6 hours (Hong et al., 2018). Second, hydrolysis using 0.05 M Tris-HCl and collagenase (100 IU). 25 mg of collagen in 5 mL of 0.05 M Tris-HCl buffer was incubated with 1 mL of collagenase at 37 ^{0}C for 18 hours (Lima et al., 2013).

MAIN RESULTS

Initially, porcine collagen DNA could not be directly detected with a porcine primer-probe even though RT-PCR is specifically designed to detect a DNA product. In addition, as Taq polymerase inhibited by hydrogen interactions in collagen, the DNA stability can be compromised (McCord, 2014). On the other hand, porcine gelatin, which is a partial hydrolysis of collagen, shows a successful amplification of porcine DNA (Nurilmala et al., 2020).

Prior to RT-PCR analysis, collagen must be hydrolyzed with enzymes and acids. This study used two types of enzymes, namely collagenase and the papain enzyme. The collagenase was incubated in 0.05 M Tris-HCl as a buffer, while the papain enzyme was incubated in 2% formic acid. The hydrolysis of particular amino acids is carried out using enzymes because enzymes are specific for cutting certain amino acids. The enzymatic hydrolysis is also processed at low temperatures so that it does not damage the structure (Selvakumar et al., 2012).

However, in this study, the pre-treatment step with papain enzyme 200 IU and formic acid could not hydrolyze collagen, thus RT-PCR could not detect it. Contrary to that, RT-PCR detected porcine collagen after pre-treatment step with 100 IU of collagenase enzymes. This is because the collagenase enzyme is a protease that recognizes Pro-X-Gly amino acid sequences. The issue arises because many amino acids repeat in collagen, with the X and Y

locations being predominantly proline/hydroxyproline amino acids. Definitively, the amino acid hydroxyproline stabilizes the protein structure, making the hydrolysis process more difficult.

Table 1 shows the Ct (Cycle of Threshold) value for collagen detection using RT-PCR. The Ct value in the porcine collagen sample was invalid because the Internal Positive Control (IPC) value was not analyzed, as well as the porcine collagen sample hydrolyzed with the papain enzyme was still not detected by RT-PCR. Nevertheless, collagen hydrolyzed with collagenase enzymes and Tris-HCl has a Ct value of 36.15. This follows the literature which explains that the maximum value is ~ 40^2.

Table 1. Result of porcine collagen DNA testing

Sample	Information	Concentration	Purity	Gene	C(t)
SKB	Porcine Collagen Sample	4.4	1.98	Porcine IPC	N/A N/A
SKB.EP	Porcine Collagen Sample, hydrolysis Papain Enzyme	3.5	1.99	Porcine IPC	34.21 N/A
SKB.EK	Porcine Collagen Sample, hydrolysis Collagenase Enzyme	5.3	2.45	Porcine IPC	36.15 30.61

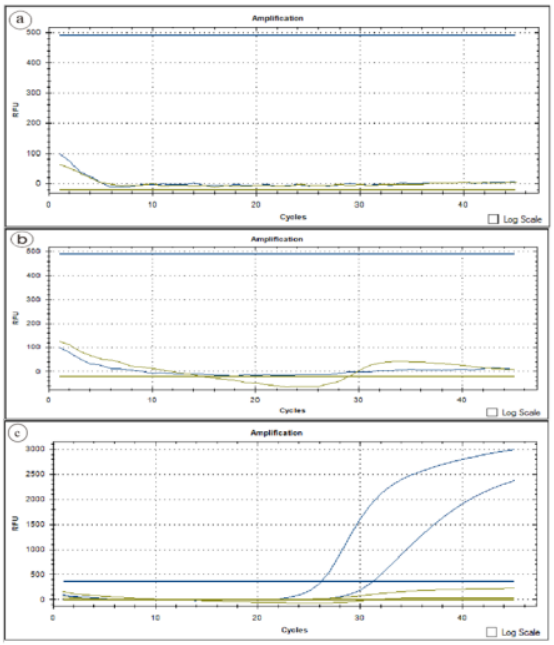

Figure 1. Amplification curves of porcine DNA detection using Real-Time PCR for a. porcine collagen; b. porcine collagen hydrolyzed with papain; c. porcine collagen hydrolyzed with collagenase.

The detection of porcine collagen using real-time PCR shows negative results for FAM and

VIC (Fig. 1). The FAM is a curve that shows porcine DNA, whereas if a positive sample contains pork, the FAM curve will rise. In that figure, the FAM curve is shown with a blue line. The VIC, on the other hand, is a curve that shows IPC DNA. The porcine collagen degraded by the papain enzyme revealed an invalid amplification curve, while the FAM curve increased. It differs from porcine collagen, which was hydrolyzed by collagenase enzymes. The real-time PCR results show that the curve is well amplified. Both the FAM and VIC curves show a positive result.

CONCLUSION

Collagen is a unique protein because it cannot be directly detected using RT-PCR, whereas gelatin, which is the result of partial hydrolysis of collagen, can be directly detected using RT-PCR. Therefore, a pre-treatment is needed to detect porcine collagen and can be included in the RT-PCR method used for halal authentication of collagen. Pre-treatment by hydrolysis using collagenase can break down the structure of certain amino acids so that the DNA can be detected using RT-PCR. In addition, the Ct value in the pig collagen sample hydrolyzed with collagenase was 36.15.

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