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ORIGINAL ARTICLE

Utilization of gel electrophoreses for the quantitative estimation of digestive enzyme papain

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Abstract SDS-PAGE densitometric method for analysis of papain in pharmaceutical formulations was developed and validated for the first time. Standard and samples were mixed with SDS sample buffer and denatured at 95 °C for 5 min and the gel was run at 20 mA and 200 V for 30–40 min in SDS-PAGE buffer. Gels were stained in Coomassie blue solution and distained by 5% methanol and 10% acetic acid. Destained gels were imaged and analyzed using the ChemiDoc™ XRS + System. Bands of papain appeared at R_f value 0.78 ± 0.03 corresponding to molecular weight 23406 Da between proteins with molecular weight 31,000 and 21,500 Da of the broad range protein standard. The generated calibration curve was used for quantitative estimation of papain in pharmaceutical formulations. The developed method was validated for precision, accuracy, specificity and robustness as described by the ICH guidelines. The proposed method gives an alternative approach for enzymes and protein analysis.
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1. Introduction

Gel electrophoresis is a method widely used for separation and analysis of macromolecules such as proteins, enzymes, DNA and RNA. In electrophoresis charged molecules move in a buffer solution by applying an electric field. Mobility is based on the charge, shape and size of the molecules. The concentration and pH of the buffer, the temperature and field strength all influence the molecular movement (Westermeier, 2005). The speed of flow is determined by the molecular weight where smaller molecules migrate faster than larger ones (Sambrook and Russel, 2001). The gel matrix acts as a molecular sieve to aid in the separation of molecules on the basis of size (Dolnik, 1997). The most commonly used materials for the separation of nucleic acids and proteins are agarose and polyacrylamide (Reddy and Raju, 2012). Polyacrylamide gel electrophoresis (PAGE) chemically cross-linked gels formed by the polymerization of acrylamide with a cross-linking agent.

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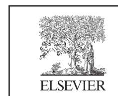

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They have a greater resolving power and can accommodate larger quantities of samples without decrease in resolution (Guilliatt, 2002). Proteins are heated with sodium dodecyl sulfate (SDS) before electrophoresis so that the charge-density of all proteins is made roughly equal. Consequently, when these samples are electrophoresed, proteins separate according to mass alone, with very little effect from compositional differences (Laemmli, 1970; Day and Humphries, 1994).

Papain is a proteolytic enzyme present in *Carica papaya* latex of the unripe fruits and used as digestive aid, meat tenderization and production of protein hydrolysates (Burdock, 1996). X-ray study revealed that papain is single polypeptide chain of 211 residues folded into two distinct parts which are divided by a cleft containing the cysteine and histidine active site (Drenth et al., 1968).

Usually the assay of enzymes is based on the measurement of the change of the concentration of substrate or product by time (Duggleby, 2001). The hemoglobin method method for the estimation of proteinase including papain is based on measurement of products released after hemoglobin digestion colorimetry (Anson, 1938). A titrimetric determination of the acid produced during the hydrolysis of benzoyl-L-arginine ethyl ester (BAEE) by papain was also developed (www.worthington-biochem).

The present study describes for the first time the quantitative analysis of papain in pharmaceutical formulations using planar SDS-PAGE. Selected formulations represented all dosage forms containing papain in the Egyptian market. Tablets, capsules and syrup prescribed as digestive aid were examined for their papain contents. The developed method was validated according to the guidelines of the International Conference on Harmonization (ICH, 1996). The developed method enables the simultaneous quantitative estimation of the enzyme in many multi component samples.

2. Materials and methods

2.1. Standards and chemicals

Standard papain was purchased from Sigma–Aldrich, St. Louis, MO, USA. Mini-PROTEAN® TGX™ gels, sample and running buffers, broad range protein standard were purchased from Biorad, California, USA.

2.2. Sample preparation

Tablets and capsules were labeled to contain either 50 or 100 mg papain. Ten tablets were weighed and grounded while contents of ten capsules were mixed and weight equivalent to two tablets or capsules was suspended in 25 mL distilled water for formulations labeled to contain 50 mg papain or 50 mL distilled water for formulations labeled to contain 100 mg papain using volumetric flasks to obtain solutions containing 4 mg/mL of papain. The obtained suspensions were filtered to separate papain and other water soluble components from the water insoluble contents. The obtained clear solutions were kept at −4 °C till time of analysis. Liquid formulation labeled to contain 80 mg of papain per 5 mL. From this solution 25 mL was diluted to 100 mL with distilled water in volumetric flask and was kept at −4 °C till time of analysis.

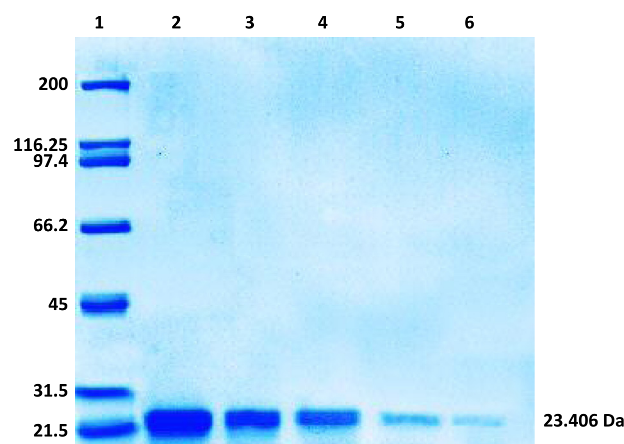

Figure 1 Chromatograms of different concentrations of standard Papain (23.406 Da, Lanes 2–6) on SDS-PAGE electrophoresis along with broad range molecular weight marker proteins (Biorad, USA) (Lane 1).

2.3. Running the gel

Enzyme samples previously prepared from standard papain and pharmaceutical formulations were added to 10 μL 2x SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 25% (v/v) glycerol, 0.01% bromophenol blue, 5% β-mercaptoethanol or 100 mM DTT) and denatured at 95 °C for 5 min. The comb was carefully removed from the gel, wells were rinsed with deionized water and the gel mounted in the SDS-PAGE apparatus. The SDS-PAGE buffer (25 mM Tris, 186 mM Glycine, 0.1% SDS) was added to the bottom and the top reservoirs. The wells have been washed with the buffer before loading the samples to exclude any trapped air bubbles. Samples were loaded to the bottom of the wells using an equipped pipette. The electrodes were connected to a power supply. The run was carried out at 20 mA and 200V for 30–40 min until the bromophenol blue has reached the lower edges of the gel.

2.4. Staining the gel with Coomassie blue

Gels were stained in a solution containing a final concentration of 0.25% Coomassie blue, 50% methanol, and 10% acetic acid, and distained by diffusion in a solution of 5% methanol and 10% acetic acid. Distaining was prolonged for 4–8 h.

2.5. Gel imaging and analysis

Distained gels were imaged and analyzed using the ChemiDoc™ XRS + System (Biorad, California, USA).

2.6. Calibration curve by SDS-PAGE-densitometric method

Accurately weighed 10 mg standard papain was dissolved in 2 mL distilled water in a volumetric flask to give 50 μg/10 μL. Dilutions from this solution were made to obtain 40, 30, 25, 20, 15, 10, 5, 2.5 μg/10 μL. Solutions were kept at −4 °C till

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