Food Research International 105 (2018) 52–58

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

# Identification of enzyme origin in dough improvers: DNA-based and proteomic approaches

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ARTICLE INFO

*Keywords:*
Dough improvers
Enzymatic co-adjuvants
Porcine pancreatic α-amylase
PCR
Shotgun proteomics

ABSTRACT

Enzymatic dough improvers (DIs) are increasingly used as baking co-adjuvants. Herein, an array of techniques, including Western blotting, PCR, electrophoresis-based and shotgun proteomics, was addressed to identify the enzymes in six commercial DI preparations. In particular, this work sought to exclude the possible undeclared use of amylolytic enzymes from porcine (or other animal origin) pancreas in DIs. PCR-amplified mitochondrial cytochrome b (mt *cyt b*) gene region and porcine pancreatic α-amylase were the targets of DNA-based and protein methods, respectively, both assuring a limit of detection lower than 0.5–0.1% (w/w). *Aspergillum oryzae* α-amylase and *Hordeum vulgare* (barley) β-amylase were the most represented enzymes in all DI samples. Although one sample was PCR-positive, none among the DIs contained porcine pancreatic enzymes. Comparative gas chromatographic analysis of fatty acids suggested that the porcine contamination might arise from hard fats of porcine origin (lard), emphasizing the need of performing analyses at the protein level when the targets are enzymes or proteins.

## 1. Introduction

Flour treatment agents, also referred to as bread improvers, dough conditioners or improvers, are additives categorized within the class of technological adjuvants, which are typically combined with cereal flours to improve the baking performance. An increasing number of dough improver (DIs) is now available for both large- and small-scale bakeries. Most of the DIs contain opportune enzymatic combinations or specific enzymes obtained from several plant or microorganism sources. Enzymes used in DIs are generally recognized as safe (GRAS) and are inactive at the end of the baking process, due to denaturation of their protein structure. Thus, according to the EU Regulation, baking enzymes can be considered as "processing aids", which need not be declared on the label (Commission regulation (EC), 2008). α-Amylase, an endoamylolytic enzyme that hydrolyzes the α-1,4-glucosidic linkages of starch, is by far the most common agent of enzymatic DI preparations. End products of α-amylase hydrolysis are soluble oligosaccharides of varying length and limit dextrins, that are a mixture of maltose, maltotriose and branched oligosaccharides of 6–8 glucose units containing both α-1,4 and α-1,6 linkages (Whitcomb and Lowe, 2007). Other glycolytic enzymes, such as debranching enzymes and β-amylases, can contribute to the starch breakdown, releasing reducing mono- and disaccharides.

To date, three endogenous α-amylase isoforms have been characterized in soft wheat grain (*Triticum aestivum*), two of which, namely TaAMY1 and TaAMY2, being the major ones (Barrero et al., 2003). Refined wheat flours retain very low amounts of α-amylase, which mainly resides in the aleuronic layer of the caryopsis before sprouting and is for the most removed during milling.

In general, high levels of α-amylase activity are detrimental to the baking quality of the flour. For instance, in late maturity α-amylase and in post-harvest sprouting wheat, the relatively high amylolytic potential has a negative impact on some quality indexes of flour, such as falling number, reducing the starch gel viscosity and impairing the formation of crumb (Ral et al., 2016). Nevertheless, bakers have long used amylolytic enzymes, especially α-amylase, either in the form of malt flour or food-grade enzyme preparations to improve the quality of dough. In fact, a controlled amylolysis facilitates dough handling and affects positively alveolation and texture of bread and baked products. The release of fermentable sugars increases the gas development during

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https://doi.org/10.1016/j.foodres.2017.10.046
Received 30 June 2017; Received in revised form 23 October 2017; Accepted 28 October 2017
Available online 07 November 2017

dough leavening, increasing loaf volume and promoting the development of the typical bread flavor as well as the formation of Maillard-related browning products in the crust (Gökmen, Açar, Serpen, and Morales, 2008; Ral et al., 2016). By altering the content and the fine structure of amylose, α-amylase also hampers starch retrogradation, delaying staling of baked goods (Miguel, Martins-Meyer, Figueiredo, Lobo, and Dellamora-Ortiz, 2013). Furthermore, enzymatic DIs compensate the loss of wheat α-amylase, thereby balancing the natural fluctuation of flour composition and the variability in bread making processes (Butt, Medhi, Munir, and Bajwa, 2000).

Recently, several journalistic reports covering the food science area roused suspicions that DIs might be supplemented with variable amounts of enzymes from porcine pancreas, or even crude porcine pancreas, as an inexpensive source of α-amylase. In principle, such a practice would not undermine the safety of baked products, but it could raise ethical concerns, for instance for vegetarian or Muslim people as well as for consumers who want to be aware about the ingredients of what they eat.

It could be argued that the incorporation of crude porcine pancreatic (PP) extracts in wheat flour might be detrimental to the bread quality, due to the presence of several proteases, which would enhance the endogenous proteolytic activity of flour, weakening the gluten network. However, controlled proteolytic depolymerization can result in positive improvements, such as a better dough workability and a desirable “gluten mellowing” (Ashgar, Anjum, and Allen, 2011). Proteolytic enzymes can be even added to flour on the purpose to achieve a proper enzymatic balance, when preparing biscuits, pastries and cookies, for which a looser gluten network is required. Thus, the inclusion of animal pancreatic extracts by the DI manufacturers is a realistic eventuality.

The aim of this work was to develop a robust and reliable analytical strategy to detect PP enzymes in DI preparations, integrating several approaches such as polymerase chain reaction (PCR), proteomics and immunochemical methods. Six commercial DI samples were analyzed to detect possible contaminations of porcine DNA markers or, alternatively, PP α-amylase at the protein level.

To the best of our knowledge, this is the first study designed to investigate the enzyme composition of DIs.

## 2. Materials and methods

All chemicals, HPLC-grade solvents, pure porcine α-amylase and porcine pancreatin were purchased from Sigma-Aldrich (St. Louis, MI, USA).

### 2.1. Samples

DI powders (approximately 50 g for samples), thereinafter indicated with progressive numbers (DI no. 1-DI no. 6), were obtained from several local bakers, without communicating them the reason of the sample collection. Commercial names of DIs and technical sheets were recorded as well, verifying that they were six different preparations. DI samples were stored at − 20 °C until use.

### 2.2. DNA isolation and PCR

DNA was extracted by using the DNeasy® mericon® Food Kit (Qiagen, Milan, Italy) according to the manufacturer's protocol. The kit combines a modified cetyltrimethylammonium bromide (CTAB) extraction with a column based purification procedure and has been optimized for DNA extraction from highly processed food material. Briefly, DI powders (200 mg) were frozen in liquid nitrogen and ground into a pre-chilled mortar. Samples were homogenized in 1 mL of food lysis buffer and digested with proteinase K for 30 min at 60 °C under constant shaking. DNA was eluted in 150 μL of 10 mM Tris-Cl, pH 8.5 and stored at − 20 °C until PCR analysis. All DNA samples were extracted in triplicates. DNA was further quantified by the Qubit™ 3 Fluorometer (Invitrogen, Carlsbad, CA, USA) using the dsDNA BR Assay Kit according to the manufacturer's instructions. PCR was performed on a PTC-100 PCR System (MJ Research, Watertown, MA, USA) in a final volume of 50 μL, containing 1 U Platinum Taq DNA Polymerase (Invitrogen), 1.5 mM MgCl2, 400 nM corresponding primers (*Cyt*-b Forward ACGTAAATTACGGATGAGTTATTCGC; *Cyt*-b Reverse GCTGTTGCTATAACGGTAAATAGTAGGAC) and 10 ng of each DNA template. After an initial denaturation at 95 °C for 5 min, 30 cycles were performed by denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s and extending at 72 °C for 30 s. A 5 μL aliquot of the resulting samples was loaded onto a 1.5% agarose gel and separated at room temperature. DNA bands were visualized by ethidium bromide staining and images acquired on a Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA, USA). PCR was also performed on DNA samples deriving from barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) flour as well as from human K562 cells as the negative controls, in order to exclude amplification cross-reactivity. PCR positive controls included DNA extracted from pork fresh meat (skeletal muscle), purchased from local butcher and immediately processed, and from pancreatin, which is a commercial mixture of hydrolases from porcine pancreas, including α-amylase, proteases with different specificity, lipases and ribonuclease.

### 2.3. Protein extraction

Proteins were extracted from 100 mg of DI samples in 1 mL of 50 mM ammonium bicarbonate (pH 7.8) containing 1 mM inhibitor of serine-proteases (Pefabloc®, Sigma). Suspension was vortexed for 5 min, sonicated for 15 min and then centrifuged (3000*g*, 15 min, 4 °C). The pellet was re-extracted twice with the same buffer and pooled supernatants were finally freeze-dried. Porcine α-amylase and pancreatin proteins were suspended using the same buffer above and used as the positive controls for α-amylase detection. Proteins in the extracts were quantified using a modified micro-Lowry assay kit (Sigma).

Proteins were also extracted from one of the improvers (i.e. DI no. 4), which had been previously assessed as free of both PP enzymes and porcine DNA by proteomics and PCR, spiked with 0.1%, 0.5%, 1%, 5% and 10% (w/w) pancreatin.

### 2.4. SDS-PAGE analysis and Western blotting

Two 8–16% gradient SDS-PAGE precast gels were (Bio−Rad, Milan, Italy) prepared and run simultaneously. Gel wells were loaded with 10 μg of DI protein extracts, 2 μg of pancreatin and 1 μg of standard α-amylase dissolved in 10 μL Laemmli sample buffer. After separation, one gel was stained with G-250 Coomassie Blue Silver, while the other was electroblotted onto 0.2 μm nitrocellulose membranes using a Trans-Blot Cell (GE healthcare, Milan, Italy) at 400 mA for 1 h at 4 °C. For Western blot analysis, the membrane was blocked for 1 h at room temperature with 5% (w/v) non-fat dry milk (Bio-Rad) in Tris-buffered saline solution with 0.05% Tween 20 (TBS-T) and incubated overnight at 4 °C with the amylase (G-10, sc-46657) mouse monoclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany) raised against amino acids 212–492 (mature polypeptide) of human amylase 2B, previously diluted 1:1000 in TBS-T.

After extensive washing with TBS-T (3 × 10 min), the membrane was incubated for 1 h at room temperature with monoclonal horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (Abcam, Cambridge, UK) diluted in 1:10,000 TBS-T, rinsed with TBS-T (3 × 10 min) and with TBS (1 × 10 min) and finally developed using the enhanced chemiluminescence ECL Prime substrate (GE Healthcare). Immunoreactive bands were visualized using X-ray film (Kodak, Chalons/Saône, France) at various exposure times ranging from 0.5 to 5 min in dark room. To determine the limit of detection (LOD) of PP α-amylase, 10 μg of protein extracts from DI no. 4 spiked with varying