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An enzyme-linked immunosorbent assay for the detection of diacetyl (2,3-butanedione)

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ABSTRACT

Diacetyl (2,3-butanedione) is an important metabolic marker of several cancers, as well as an important off-flavour component produced during fermentation. As a small molecule in a complex mixture with many other analytes, existing methods for identification and quantitation of diacetyl invariably involves a chromatographic separation step followed by signal integration with an appropriate stoichiometric detector. Here we demonstrate that the chemical reaction of diacetyl with a 1,2-phenylenediamine derivative yields a chemical adduct, 1,4-quinoxaline which can be conjugated on BSA. The BSA-diacetyl adduct can be used to select an adduct-specific monoclonal antibody in a Fab-format from a 45-billion member phage-display library. The availability of this antibody allowed the development of an enzyme-linked immunosorbent assay for diacetyl, based on the 1,4-quinoxaline competition for the antibodies with the diacetyl adduct immobilized on the plate. The described ELISA assay can detect the captured diacetyl in micromolar concentrations, both in water samples and in cell culture medium.

Introduction

Diacetyl (DA, 2,3-butanedione) is a volatile flavour compound, naturally produced by bacteria and yeast, and commonly known for its characteristic “butter-scotch” aroma [1]. For its essential role in enriching the buttery aroma in fermented foods and beverages, it historically received great attention from the food industry [2]. Lactic acid bacteria (as *Oenococcus oeni* or various other species of *Lactobacillus* and *Pedicococcus*) are responsible for diacetyl release as an intermediary compound in the metabolism of citric acid during malolactic fermentation [1, 3, 4]. Vicinal diketones (diacetyl and 2,3-pentanedione) are even produced by yeast in the branched-chain amino acids biosynthetic pathway (BCAA, isoleucine, leucine, and valine) as by-products from the intermediate alpha-acetolactate, decarboxylated into diacetyl [5-7]. Sensory threshold in wine and beer highly depends on the type and style of wine and beer, ranging from a positive trait (as in dark beers and red wines) to a particularly undesirable off-flavour in lager beers [1]. In the food industry, investigations about the genetic diversity of diacetyl-producing strains have the double role of identifying new traits or focusing on DA reduction for commercial applications [8-11].

In the scientific community, diacetyl has gained increasing interest for its known toxicity. As a volatile hazardous compound, it has been identified as a causing agent for lung disease [12]. Exposure to diacetyl has been related to onset of *bronchiolitis obliterans* lung diseases, especially known as “popcorn worker's lung” for its manifestation in workers in popcorn manufacture [13]. Inhalation of diacetyl results in acute toxicity for the airways and lung epithelium [14], and DA toxicity also appears in the form of covalent adduct formation with arginine residues leading to immunological response [15], or chronic response through accelerating β-amyloid aggregation associated with Alzheimer's disease [16]. Moreover, mutagenic properties of diacetyl have recently been exploited, showing that DA can interact with deoxyguanosine on DNA, affecting DNA ternary structure and cell survival [17].

For its toxicity, as well as for its implication as an aroma compound, diacetyl presence and threshold have to be accurately determined in biological samples and during industrial processes. Detection methods should allow selective and precise diacetyl level determination. Several existing methods for DA determination involve derivatization of the vicinal diketones, diacetyl and pentanedione, to the quinoxaline derivatives, followed by detection through gas-chromatography coupled with mass-spectrometry (GC-MS) [18,19], high performance liquid chromatography (HPLC)

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Abbreviations

Anti-DAA	anti-diacetyl adduct antibody
Boc_2O	di-*t*-butyl dicarbonate
BSA	bovine serum albumin
BSA-DAA	BSA-conjugate of the diacetyl adduct
BSA-PDA	BSA-conjugate of the pentanedione adduct
DA	diacetyl
DAA	diacetyl adduct
DCM	dichloromethane
DMAP	4-(dimethylamino) pyridine
DMEM	Dulbecco's Modified Eagle's Medium
DSC	N, N'-disuccinimidyl carbonate
ESIMS	electrospray ionization mass spectrometry
FBS	fetal bovine serum
Kdo	keto-deoxy-octulosonic acid
HuCAL®	human combinatorial antibody library
MALDI-TOF-MS	matrix assisted time of flight mass spectrometry
NHS	*N*-hydroxy-succinimide
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline plus 0.05% (v/v) Tween® 20
PDA	pentanedione adduct
PPB	part per billion
TEA	triethylamine
TFA	trifluoroacetic acid

[20,21], differential pulse polarography [22], or adsorptive stripping voltammetry [23]. Another analytical method for vicinal diketones in fermented beverages relies on their volatile nature by gas chromatography analysis of the sample headspace [24,25].

By non-invasive GC-MS analysis of exhaled breath, potential marker compounds for lung cancer have been identified [26], including diacetyl in the list of volatile compounds hypothetically useful for diagnostic purpose. Besides chromatographic techniques, colorimetric methods for diacetyl determination have been developed since the Westerfield method [27,28] in blood, where creatine reacts with DA in the presence of α-naphthol developing a chromogenic compound. Alternatively, a two-step reaction with isoniazide and zirconium allows the vicinal diketones to form fluorescent complexes, detectable in food samples [29]. Recently, ruthenium probes were developed [30] with the aim of detecting diacetyl in early-stage cancer cells. After reaction with DA, the ruthenium compounds turn luminescent and diacetyl detection seems feasible at physiological conditions in culture cell media. To date, the disadvantage of some of these methods is that they require reactions with compounds which cannot distinguish diacetyl from other vicinal diketones, causing the interference to invalidate diacetyl detection. Diketones derivatization or other separation and distillation procedures contribute to difficulties or inaccuracy in the analysis. Fluorescent diacetyl probes include rhodamine hydrazide [31], colorless in its free form, but capable of giving a fluorescent derivative after reaction with DA. Aiming for diacetyl detection in physiological samples, such as cancer cells, assay selectivity and applicability are two main requirements, while too extreme assay conditions, as the acidic pH needed for the DA determination with the rhodamine probes (around pH 3) [31], represent a limit for the assay.

Assay selectivity, for detection of small molecules such as diacetyl, is achievable by the development of assay methods with specific capture of the molecule of interest and unique selection of the formed complex. A new approach for small-molecule analysis using specific antibodies was developed in our laboratory for the Kdo (Keto-deoxy-octulosonic acid), a monosaccharide present in bacterial cell-surface lipopolysaccharides [32]. Reaction of Kdo with a 1,2-phenylenediamine derivative forms a quinoxalinone adduct, which can be specifically recognized by antibodies raised against the BSA-coupled adduct. By immobilizing the capture compound on a solid support and reacting it with the target molecule, Kdo, adduct formation allows detection by specific antibodies in an ELISA format.

Here we developed an ELISA assay for detection of the small molecule diacetyl in solution. The assay is based on a chemically synthesized 1,2-phenylenediamine attached to a symmetrical linker. The 1,2-phenylenediamine derivative reacts with diacetyl, giving the chemical adduct 1,4-quinoxaline that can be selectively recognized with HuCAL® antibodies found by phage display. Taking advantage of selective antibody recognition, a competitive ELISA assay is designed, which specifically detects diacetyl at physiological conditions in complex samples among other α-carbonyls.

Material and methods

Reagents

Bovine serum albumin was purchased from Calbiochem (BSA, fraction V, crystalline, 12,657), *o*-phenylenediamine (78,410), H_2O_2 (H1009), Dulbecco's Modified Eagle's Medium (D6046), fetal bovine serum (FBS, F2442) and gentamicin (G1397) were purchased from Sigma-Aldrich.

Synthesis of the methyl esters of diacetyl-adduct (DAA) and pentanedione-adduct (PDA) and preparation of the corresponding BSA-conjugates (BSA-DAA and BSA-PDA)

The 1,2-phenylenediamine derivative used as capture molecule (linker diamine, Scheme 1), with a symmetric linker to avoid isomeric mixture when asymmetric diketones like pentanedione are involved, was synthesized in 5 steps from 4-piperidone and glutaric acid monomethylester chloride. Reaction of the linker diamine with diacetyl under standard conditions gave the 1,4-quinoxaline adduct (DAA). After hydrolysis of the methyl ester and NHS-activation of the acid, the compound was conjugated to the lysines in BSA in phosphate buffer at pH 7,5 to give BSA-diacetyl adduct (BSA-DAA). MALDI-TOF-MS analysis of the conjugate found m/z 70,530, that corresponds to an incorporation of ~11 diacetyl adducts per BSA [33,34] (Fig. 1) This conjugate was used for antibody screening and selection. The BSA-pentanedione-adduct (BSA-PDA), used for counter selection of the antibodies, was synthesized from the pentanedione adduct (PDA) according to the same procedure as for BSA-DAA, resulting in incorporation of 11 pentanedione adducts per BSA (m/z 70,380 by MALDI-TOF- MS, Fig. 1).

See Ref. [35] for all experimental procedures.

Preparation of 1,2-phenylenediamine capture molecule

Due to the instability of the 1,2-phenylenediamine (linker diamine) the aromatic amino groups were Boc-protected followed by purification by silica gel chromatography. Prior to the capturing experiments the amines were liberated by treatment with TFA, followed by drying under a stream of argon, Scheme 2 and [35].