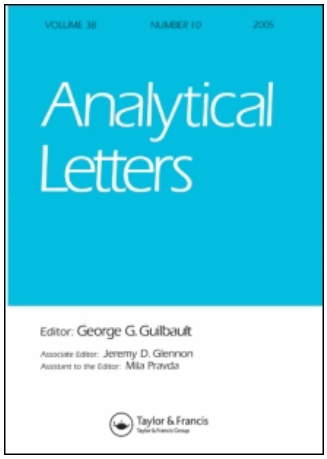

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Determination of Damaged Starch in Wheat Flour Using an Electrochemical Bienzyme Maltose Probe

E. Marconi [a]; C. Baldino [a]; M. C. Messia [a]; R. Cubadda [a]; D. Moscone [b]; G. Palleschi [b]
[a] DISTAAM, Università del Molise. Via De Sanctis, Campobasso, 86100. Italy
[b] Dipartimento di Scienze e Tecnologie Chimiche, Università di Roma Tor Vergata. Via della Ricerca Scientifica, Roma, 00133. Italy

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DETERMINATION OF DAMAGED STARCH IN WHEAT FLOUR USING AN ELECTROCHEMICAL BIENZYME MALTOSE PROBE

Key words: damaged starch, wheat flour, bienzyme maltose biosensor

E. Marconi, C. Baldino, M.C. Messia, R. Cubadda

DISTAAM, Università del Molise, Via De Sanctis, 86100 Campobasso, Italy.

D. Moscone and G. Palleschi

Dipartimento di Scienze e Tecnologie Chimiche, Università di Roma Tor Vergata, Via della Ricerca Scientifica, 00133 Roma, Italy.

ABSTRACT

The development of an electrochemical biosensor based on a bienzyme maltose probe and a third enzyme α-amylase in solution is reported for the rapid and inexpensive determination of damaged starch. Analytical parameters, such as probe stability, pH, temperature and response time, were optimised. Damaged starch was measured in the range of 5×10^{-6} - 5×10^{-4} mol/L as maltose produced by the enzymatic reaction and the detection limit was calculated according with the

free maltose and/or glucose in the sample. The damaged starch was determined in different wheat flours, and the data significantly correlated with those obtained using a reference procedure ($r^2 = 0.994$; $P \leq 0.0001$). In addition the results showed a comparable precision (CV < 5 %). This method is rapid, inexpensive and friendly for unskilled operators.

INTRODUCTION

The milling process of wheat produces a partial damage of starch granules in the flour caused by forces of compression, impact, shear or attrition. The amount of damage varies with the severity of the milling process and the hardness of the wheat kernel. Since the damaged granules absorb more water and are more susceptible to enzymatic hydrolysis than undamaged starch, they influence the water absorption, rheology, gassing power of a dough, and the crumb texture and crust colour of the baked products. Damaged starch is also a substrate for amylases which provide fermentable carbohydrate that is utilized by yeast. Therefore a certain degree of damage is in part desirable to promote the fermentation activity during bread making, while an excessive damage promotes rapid starch hydration with resulting accelerated enzyme activity, sticky dough and problems in bread slicing[1].

In pasta making the degree of starch damage favours the Maillard Reaction (MR) during the drying process, with an increase of unavailable lysine and dark colour[2]. The reducing sugars, produced by amylolysis of damaged starch, are in fact the limiting reactants in semolina for the development of MR during pasta drying at high temperature[3].

The assessment of starch damage in flour or semolina is, therefore, of great importance for the milling, baking and pasta making industries.

During the past years numerous methods for starch damage measurement in flour have been developed.

An improved enzymic spectrophotometric method (kit) was recently standardized[4] and adopted by the American Association of Cereal Chemists[5]. An automated non-enzymatic method, the Chopin SD4 (Seedburo Equipment Co., Chicago IL), was also recently introduced[6]. Other analytical methods, such as Near-Infrared Reflectance (NIR)[7] and RP HPLC[8] have also been reported. Several publications describing the comparison between enzyme and non-enzymatic methods[9-13] agree that enzymatic methods are preferred for their better sensitivity.

All the above mentioned methods are often complicated and/or require expensive reagents and/or instrumentation and skilled operators.

The aim of this study was to develop an enzymatic method based on a maltose electrode probe and the enzyme α-amylase in solution.
The reactions involved are:

$$\text{Damaged starch} \xrightarrow{\alpha\text{-amylase}} \text{Maltose} + \text{Dextrin} \qquad (1)$$

The damaged starch granules have, in fact, a characteristic of easy hydrolysis by amylolytic enzymes: in particular, the α-amylase allows a near complete hydrolysis of damaged granules with minimum breakdown of native granules[1]. This hydrolytic process is biphasic: the initial rapid phase represents the hydrolysis of damaged granules and the second phase the slow hydrolysis of native starch[9]:

$$\text{Maltose} + \text{Dextrins} \xrightarrow{\text{amyloglucosidase}} \text{Glucose} \qquad (2)$$

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{Gluconic acid} + H_2O_2 \qquad (3)$$

The hydrogen peroxide produced in the third reaction is oxidized by an amperometric hydrogen peroxide platinum probe held at +650 mV (versus Ag/AgCl). The output current is correlated to the glucose present in the sample (reaction 3), then to the amount of maltose (reaction 2) and finally to the damaged starch (reaction 1).

The amyloglucosidase and glucose oxidase enzymes were immobilized on the electrode surface, while the α-amylase was added in solution. Different types of enzymes and membranes and different procedures of immobilization were carried out in order to find the best operational conditions. In addition, calibration curves of glucose, maltose and starch damage were carried out to optimize analytical parameters such as pH, buffer, temperature, probe lifetime and response time. Control wheat flours with different levels of damaged starch were analysed both through the biosensor and the standard reference procedures.

EXPERIMENTAL

Materials

Six wheat flour samples (WF1-WF6), one wheat reference flour (WRF) from MegaZyme Pty Ltd and one wheat native starch (WNS) with a wide range of starch damage (from 2 to 7% flour weight, which covers the entire range likely to be experienced in commercial wheat flour and semolina) were used.

α-Amylase (type X-A: fungal, crude from *Aspergillus orizae,* Sigma, EC 3.2.1.1; 40 units/mg solid), amyloglucosidase (AMG) from *Aspergillus niger* (Sigma; EC 3.2.1.3; 51 units/mg solid) and glucose oxidase (GOD) from *Aspergillus niger* (Sigma, Type VII; EC 1.1.3.4; 176,000 units/g solid).

The enzymatic assay kit (as reference method) was supplied by Megazyme Pty Ltd, North Rocks, Australia. Each assay kit contained the following enzymes: α-amylase (EC 3.2.1.I.) from *Aspergillus niger* (1,000 U/ml, in 3.2 M ammonium sulphate), amyloglucosidase (EC 3.2.1.3) from *Aspergillus niger* (200 U/ml, in 3.2 M ammonium sulphate), glucose oxidase-peroxidase-4aminoantipyrine, glucose determination reagent (GOPOD) supplied as a freeze-dried powder; wheat reference flour (WRF) with a starch damage level of 6.2%.

All other chemicals were of analytical-reagent grade (Sigma Chemical Co., St Louis, MO).

Immobilon AV affinity membrane™ 0.65 μm pore size, 125 μm thickness was from Millipore Corp., Bedford, MA; Pall Immunodyne immunoaffinity membrane™ was from Pall Corp, Glen Cove, NY; Nylon net support was obtained from a local store; chemically activated Nylon net used for covalent bonding of the enzymes, 120 mesh, was from A. Bozzone, Appiano Gentile, Italy. Cellulose acetate membrane (approx MW cut off 100) was prepared in our laboratory according to Palleschi *et al* 1986[17]. Polycarbonate membrane, 0.03 μm pore size, 6 μm thickness was from Nuclepore, Pleasanton, CA.

Apparatus

Electrochemical measurements were carried out with an ABD amperometric biosensor detector from Universal Sensors, Metaire, LA. The electrochemical cell consisted of a hydrogen peroxide probe from Universal Sensors. Currents were recorded with a Linseis L6512 recorder (Selb, Germany). Temperature studies were performed with an Haake thermostat Model D8 (Berlin, Germany) using a double-wall glass beaker. Spectrophotometric measurements were carried out using a Varian Mod Cary 1E UV-Visible Spectrophotometer.

Enzyme Immobilization Procedures

The Immobilon membrane was prepared by cross linking the AMG and GOD enzymes with RSA and glutaraldehyde and then covalently bonding on pre activated membrane following the procedure reported by Villarta *et al* 1993[14].

17 units of AMG, 17 units of GOD, 0.05 mg BSA and 1 μL of 0.25% glutaraldehyde were dissolved in 10 μL of 0.5 M KH_2PO_4, pH 7.4 and placed on the membrane using a micropipette.

The co-immobilization of GOD and AMG on the Pall membrane was carried out asymmetrically following the procedure reported by Penguin et al 1989[15].

The immobilization of GOD and AMG on nylon net membrane was carried out following two different procedures: chemical activation of nylon[16] and the

BSA-glutaraldehyde[14]; in the second procedure the nylon net was used only as support.

Probe Assembly

The maltose probe was assembled by placing onto an inverted electrode jacket, in the given order, the following membranes: cellulose acetate membrane, the enzymatic membrane with GOD/AMG enzymes immobilized, and the polycarbonate membrane.

The. membranes were then secured to the electrode jacket with an O ring. The jacket was filled with the appropriate electrode filling solution, and the combination working/reference electrode was inserted into the jacket and screwed in place. The working electrode was pressed tightly against the inner membrane. A constant potential of +650 mV was applied to the Pt working electrode.

Measurement Procedures

Proposed method

The maltose probe was immersed in a double wall glass beaker containing 3 mL of acetate buffer 0.1 M, pH 5.0 and allowed to equilibrate to 25°C. The following buffers were also tested at different pHs: citrate buffer 0.1 M, pH 4.0 - 5.0; acetate buffer 0.1 M, pH 4.5 - 5.5; phosphate buffer 0.1 M, pH 5.5 - 7.5; borax buffer 0.1 M, pH 8.0 - 9.0. After 5 min, a stable current baseline was observed. Aliquots of maltose or glucose standard solutions were then added, and the change in current was recorded after 3 min.

Starch damage determination

First step: to an appropriate amount (100 mg) of sample was added 1.0 mL of pre-equilibrated (25°C) α-amylase (50 U/mL). The α-amylase (Sigma) was previously purified in order to remove the impurity found (glucose, maltose). It was therefore dialyzed as follows: 1 ml of α-amylase solution, containing 2,000 units, was transferred into a dialysis tube Spectra/Por Membrane 15,000 MW cut-off

(Spectrum, Houston, TX) which was then sealed and immersed into a 1 L solution of 0.1 M phosphate buffer, pH 7.0.

The sample was immediately stirred on a vortex mixer for 5 sec and then incubated at 40°C for exactly 10 min. Then 5.0 mL of diluted sulphuric acid (0.2% v/v) was added to terminate the reaction, and the sample was filtered (Whatman No. 1).

Second step: aliquots of the filtered sample (10 μL, if necessary diluted in order to assure that the current response was in the linear range of the probe) were added into 3 mL acetate buffer where the electrode probe was equilibrated. The change in current related to H_2O_2 produced was recorded after 3 min.

Reference method

The first step of the reference procedure is similar to that described for the biosensor procedure except for the type of α-amylase used (MegaZyme).

Second step: aliquots of the filtered solution (0.1 mL) were incubated with amyloglucosidase solution (0.1 mL, 2 U) for 10 min at 40°C. GOPOD reagent (4 mL) was then added to each tube, mixed on a test-tube stirrer, and the incubation was continued for another 20 min at 40°C. The absorbance at 510 nm was then measured.

Determination of free glucose plus maltose

The free glucose plus maltose in the wheat flour was determined by the. biosensor procedure after the addition of the diluted acid solution instead of α-amylase in order to stop the activity of endogenous enzymes. Then an aliquot of this solution was injected in acetate buffer where the probe was equilibrated and the free glucose (plus maltose) was measured.

Amylolytic activity of endogenous enzymes

The amylolytic activity of endogenous enzymes was calculated by the ratio between the free maltose plus glucose produced by the activity of these endogenous

enzymes (determined as above without adding the acid solution) and the starch damage content.

RESULTS AND DISCUSSION

Optimization of Analytical Parameters

Bienzyme immobilization

The calibration curves for glucose and maltose using AMG/GOD enzymes immobilized by four different procedures are shown in Fig. 1. In both curves, the highest sensitivity was reached using Immobilon AV affinity membrane with BSA/glutaraldehyde procedure.

pH study

The effect of pH on the maltose probe response was evaluated using four buffer systems with pH values ranging from 4.0 to 9.0 and is reported in Fig. 2. The optimum sensitivity of the probe was reached with acetate buffer from pH 4.5 to 5.5 with the maximum value at pH 5.0.

Temperature study

Temperature study was carried out varying the temperature from 20 to 70°C. The relative results are reported in Fig. 3. The best response for the enzyme activity was obtained between 45 and 55°C, while the activity dropped at 70°C when protein denaturation occurred. The temperature selected was 25°C because at this temperature we obtained the best signal/noise ratio.

Calibration curves of glucose and maltose

The glucose and maltose calibration curves obtained with all parameters optimized (Immobilon membrane with BSA/glutaraldehyde, acetate buffer 0.1 M at pH 5.0, temperature 25°C) are reported in Fig. 4. This figure shows that glucose

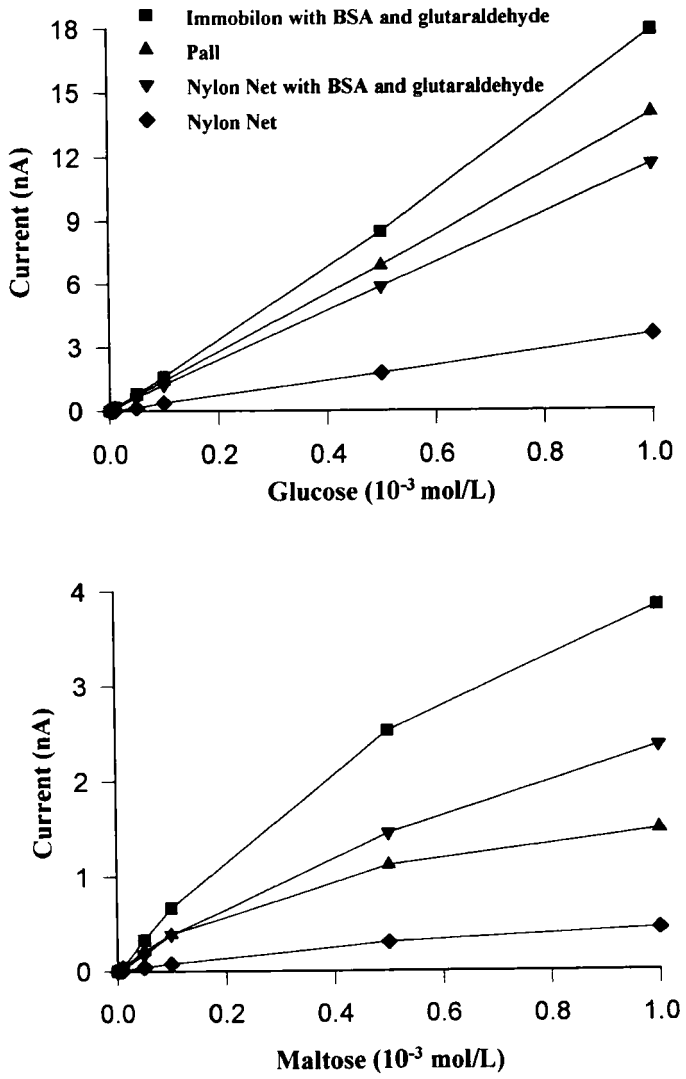

FIG. 1. Calibration curves for glucose (top) and maltose (bottom) using AMG/GOD immobilized on different types of membrane; phosphate buffer 0.1 M pH 7.4 and t = 25°C.

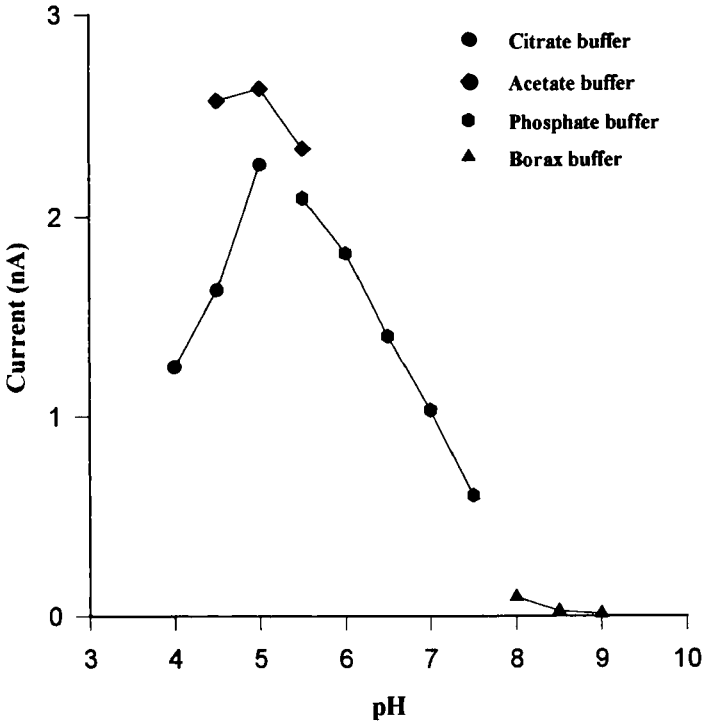

FIG. 2. pH profile of the maltose probe using different buffers 0.1 M with different pHs, AMG/GOD immobilized on Immobilon with BSA and glutaraldehyde, maltose concentration of 10^{-4} mol/L and t = 25°C.

and maltose can be detected in a linear range from 10^{-6} to 10^{-3} and from 5×10^{-6} to 5×10^{-4} respectively.

Probe lifetime

The probe lifetime during working conditions at 25°C and at 4°C during dry storage is reported in Table 1. The probe showed good stability, retaining about 100% of its activity after 20 days and 60% after 40 days.

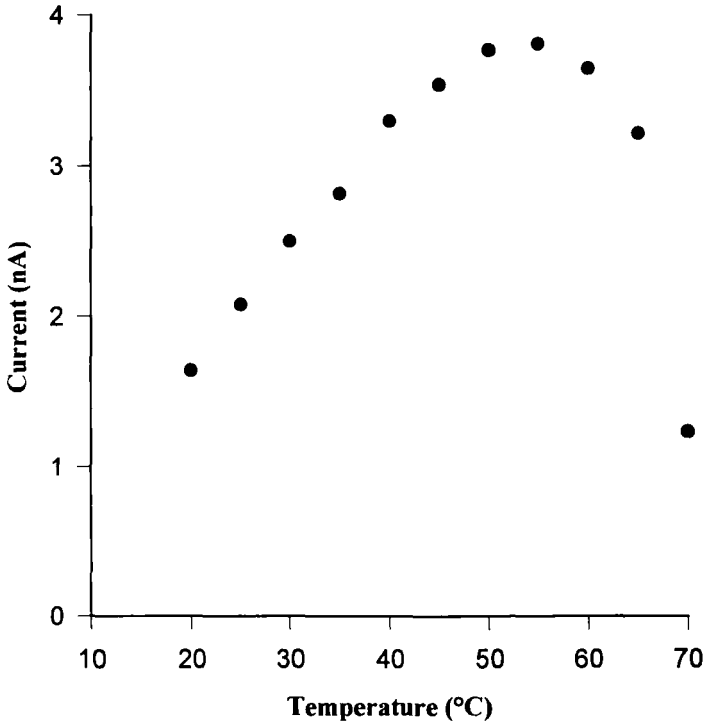

FIG. 3. Temperature profile of the maltose probe using AMG/GOD immobilized on Immobilon with BSA and glutaraldehyde; acetate buffer 0.1 M pH 5.0 and maltose concentration 10^{-4} mol/L.

Interferences study

Our probe was assembled with a cellulose acetate membrane (100 mwco) which was already used in previous work[17]. So the common electrochemical interferences as ascorbic acid and uric acid did not interfere when injected in the buffer solution where the electrode was equilibrated. Also the matrix gave negligible enzymatic or electrochemical interferences as demonstrated from the recovery studies. In fact standard injections of maltose in three different wheat

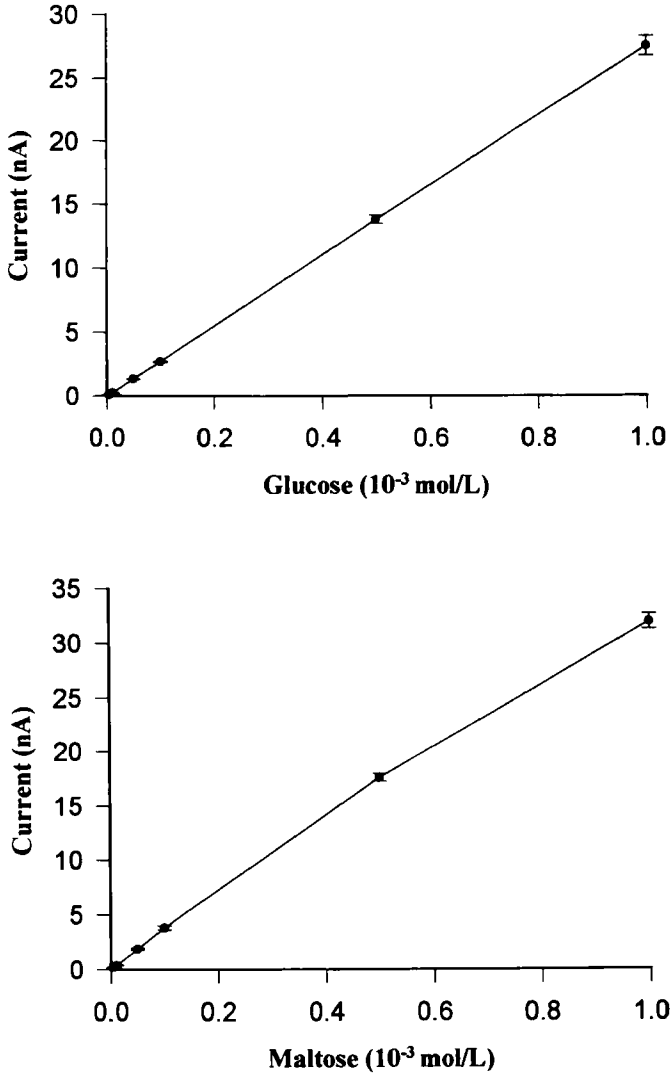

FIG. 4. Glucose (top) and maltose (bottom) calibration curves using AMG/GOD immobilized on Immobilon with BSA and glutaraldehyde; acetate buffer 0.1 M pH 5.0, maltose concentration 10^{-4} mol/L and t = 25°C.

TABLE 1

Operational Lifetime of the Maltose Probe (maltose concentration 10^{-4} mol/L)

Days	Current (nA)	Rel. enzyme activity (%)
1	1.39	100
3	1.40	100
5	1.46	105
10	1.40	101
20	1.34	97
30	1.13	81
40	0.84	61

TABLE 2

Recovery Studies of Maltose in Different Wheat Flour Samples

Sample	Present 10-3 g/L	Added 10-3 g/L	Expected 10-3 g/L	Found 10-3 g/L	Recovery %
A	2.30	1.00	3.30	3.32	100
		2.00	4.30	4.65	108
B	2.60	3.00	5.60	5.55	99
C	5.35	2.50	7.85	7.80	99
		5.00	10.35	9.90	96

flour samples (as shown in Table 2) gave a recovery of maltose in the range of 96-108%.

Analysis of wheat flour samples

The results of the determination of damaged starch, obtained with different wheat flour samples, showed a good correlation ($r^2 = 0.994$, $P \leq 0.0001$) with the reference procedure (Fig. 5). The regression equation was $DS = 73.27c + 0.077$ where DS = damaged starch and c = current (nA).

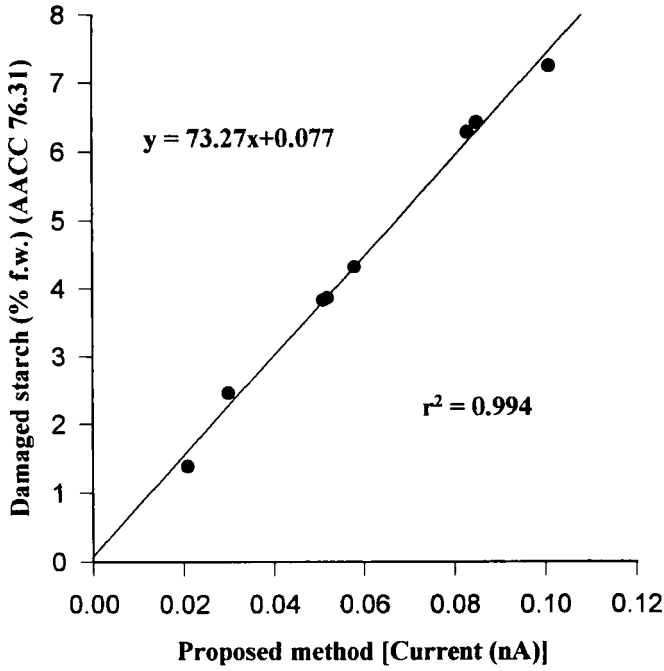

FIG. 5. Correlation between the results of different wheat flour samples obtained with the reference and the biosensor procedures.

The Relative Standard Deviation (RSD) of the proposed method (3 determinations for each sample) ranged from 0.0 to 3.4% and was similar to that of the reference procedure (0.0 to 4.5%) as reported also by other authors[9,11].

The complete time of analysis of the innovative procedure was 20 min versus 50-55 min of the standard procedure[11-13]. Moreover our method can be readily automated with a flow injection apparatus, making it highly suitable for a large number of tests.

From an economical point of view the biosensor method is more convenient than the reference procedure, because it uses low amounts of reagents and enzymes.

TABLE 3

Free Maltose Plus Glucose, Maltose Plus Glucose Produced by Endogenous Enzymes, Damaged Starch and Amylolytic Activity of Different Wheat Flour Samples

Sample	Free maltose + glucose g/100g fw	Maltose + glucose* g/100g fw	Damaged starch g/100g fw	Amylolytic activity** AU
WF1	0.1	0.6	2.3	26
WF2	0.2	1.1	3.8	29
WF3	0.1	0.7	3.9	18
WF4	0.2	1.2	6.4	20
WF5	0.2	2.3	7.3	32
WRF	0.2	1.1	6.2	1.7

*produced by endogenous enzymes
**calculated as the ratio of maltose + glucose and damaged starch × 100
AU=Arbitrary Units

The amylolysis activity of endogenous enzymes (α and β amylases) of the different wheat flour samples is reported in Table 3. The WF5 sample showed the highest amylolytic activity, 32 AU, while the WRF the lowest value, 17 AU. The low value of the reference material was expected, since this type of material should be, for its nature, very stable from an enzymatic, chemical and microbiological point of view.

CONCLUSIONS

This method, therefore, is a promising approach to a fast and accurate determination of damaged starch with a simple and rapid procedure using low amounts of sample, enzymes and reagents, inexpensive instrumentation and unskilled operators. In addition it allows an easy determination of the amylolytic activity of endogenous enzymes.

Proceedings of this paper were presented at the 10th International Cereal and Bread Congress -International Association for Cereal Science and Technology (ICC)- Porto Carras, Greece, June 9-12, 1996[18] and at the AAA BIOTEC Congress "Advanced Biotechnologies for Agriculture, Nutrition and Environment", Ferrara, Italy, October 8-11, 1996[19].

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