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Neural Network Inference of Molar Mass Distributions of Peptides during Tailor-Made Enzymatic Hydrolysis of Cheese Whey: Effects of pH and Temperature

Gilson A. Pinto · Raquel L. C. Giordano · Roberto C. Giordano

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Abstract The fine-tuning of the enzymatic hydrolysis of proteins may provide a pool of peptides with predefined molar mass distributions. However, the complex mixture of molecules (peptides and amino acids) that results after the proteolysis of cheese whey turns unfeasible the assessment of individual species. In this work, a hybrid kinetic model for the proteolysis of whey by alcalase®, multipoint-immobilized on agarose, is presented, which takes into account the influence of pH (8.0–10.4) and temperature (40–55 °C) on the activity of the enzyme. Five ranges of peptides' molar mass have their reaction rates predicted by neural networks (NNs). The output of NNs trained for constant pH and temperatures was interpolated, instead of including these variables in the input vector of a larger NN. Thus, the model complexity was reduced. Coupled to differential mass balances, this hybrid model can be employed for the online inference of peptides' molar mass distributions. Experimental kinetic assays were carried out using a pH-stat, in a laboratory-scale (0.03 L) batch reactor. The neural-kinetic model was integrated to a supervisory system of a bench-scale continually stirred tank reactor (0.5 L), providing accurate predictions during validation tests.

Keywords Cheese whey proteolysis · State inference · Immobilized alcalase · Neural networks · Hybrid model · Enzymatic hydrolysis

NOTATION

BAEE	*N*-benzoyl-L-arginine ethyl ester
C_i	Mass concentration of the pseudocomponent i ($g_{Protein}\ L_{Suspension}^{-1}$)
C_5^F	Mass concentration of the reactor feed, concentrated cheese whey ($g_{Protein}\ L_{Suspension}^{-1}$)
E	Concentration of alcalase® ($U_{BAEE}\ L^{-1}$)
MM_i	Molar mass of pseudocomponent i (Da)
q_F	Volumetric flow rate of the reactor feed ($L\ min^{-1}$)

G. A. Pinto · R. L. C. Giordano · R. C. Giordano (✉)
Departamento de Engenharia Química, Universidade Federal de São Carlos (UFSCar), Rodovia Washington Luis, km 235, CP 676, 13565-905 São Carlos, São Paulo, Brazil
e-mail: roberto@power.ufscar.br

q_C Volumetric flow rate of base used to control the reaction pH (L min^{-1})
r_i Reaction rate of pseudocomponent i ($g_{Protein}\ U_{BAEE}{}^{-1}\ min^{-1}$)
T Temperature of reaction (°C)
V Volume of the system, that is, concentrated cheese whey plus immobilized alcalase® (L)

Introduction

Cheese whey is the main by-product of the dairy industry. Whey corresponds to 85–95% of the milk volume and retains 55% of its nutrients [1]. Reuse of cheese whey has a two-sided motivation: taking advantage of its nutritional contents while avoiding emission of a strong pollutant, with a biochemical oxygen demand (BOD) of approximately 35,000 mg O_2 L^{-1} [2, 3].

Whey typically contains around 50 g/L of lactose and 7 g/L of proteins, the main ones being α-lactalbumin (16% w/w), β-lactoglobulin (49%), bovine serum albumin (5%), and immunoglobulins (10%). The world production of whey is over 145 million tons, from which 60% is recovered by several methods and 40% discarded directly, without previous treatment. Thus, obtaining products with higher commercial value from this residue seems to be an attractive solution for an environmental problem [4].

Those figures show that lactose is mainly responsible for the high BOD of whey, and establishing alternatives for the use of this sugar is an important task. Nevertheless, the protein fraction of whey should not be disregarded [5], and it is the focus of this work. Whey protein concentrates, obtained via micro- and ultrafiltration, can be submitted to sequential-controlled hydrolyses, using proteases. For example, hydrolysis with alcalase® may produce a pool of small (mostly di- and tri-) peptides, which is proper for parenteral feeding [6]. Small peptides (<2,000 Da), produced by the hydrolysis of casein by alcalase, have been used to enrich fruit drinks since 1976 [7].

In an industrial process to produce enzyme-hydrolyzed whey, it is important to work with stabilized enzymes, which may support higher operating temperatures for long periods, thus avoiding microbial growth within the reactor. On the other hand, immobilization of the enzyme would prevent autolysis, allowing the continuous operation of the reactor for long periods. Consequently, enzyme stability is a key factor for this process.

The existence of several covalent links between residues of the enzyme and an activated support (multipoint covalent attachment) may exert a very important effect on the stability of enzymes [8]. Multipoint attachments may increase the rigidity of the enzyme molecule, hence inducing a higher resistance to small conformational changes caused by heat, organic solvents, denaturing agents, and so forth. The selection of a suitable immobilization system (in this case, glyoxyl-agarose) and of adequate reaction conditions (e.g., high degree of activation of the support and prolonged immobilization time spans) enables the preparation of highly stabilized alcalase derivatives, approximately 500-fold more stable than the dialyzed soluble enzyme [9]. This is due to the several covalent links that are created between enzyme and support [10–12]. An in-depth discussion of the advantages of glyoxyl-agarose supports may be found in the work by Mateo et al. [13], and the kinetics of the hydrolysis of whey protein using glyoxyl-agarose-alcalase derivatives was studied by Tardioli et al. [14].

This work addresses the kinetic modeling of the enzymatic reaction that yields a tailor-made pool of small peptides—the alcalase® hydrolysates. Monitoring of this reaction, with real-time inferring of the molar mass distribution of the product, is very important for the fine-tuned control and optimization of the reactor operation. The detailed description of all the reactions occurring in this system is not feasible, however, not only because of the

complexity of the substrate, but also due to the number of possible products (peptides) that are generated by this endoprotease of low specificity.

Models that quantify the rate of the enzymatic depolymerization of complex molecules have to rely essentially on simplified approaches [15, 16]. The classical approach for this problem uses the "degree of hydrolysis" as process variable and the concentration of "hydrolysable bonds" as a pseudosubstrate within simple empirical rate equations or, alternatively, Michaelis–Menten expressions [6, 17, 18]. This approach, however, provides no information concerning the molar mass distribution of peptides formed during the reaction. The literature reports some attempts to circumvent this difficulty. Shi et al., studying proteolysis of bovine serum albumin by trypsin, lump the product in four molar mass ranges and fit an empirical rate equation for each of these pseudocomponents at different temperatures [19]. Mota et al. characterized the hydrolysis by trypsin of whey concentrates using multivariate data analysis [20].

Our substrate, sweet cheese whey, is a mixture of proteins, amino acids, and peptides resulting from the coagulation of casein during the cheese manufacturing process [1]. Hence, the variability of the composition of this substrate is high, depending not only on the upstream process conditions, but also on climate and seasonal factors that affect the original characteristics of the raw material, bovine milk. A methodology to assess the progress of the proteolysis reactions, which would be flexible and easily retunable, with low experimental effort, would be very convenient in this case.

Systems identification is one of the most popular applications of neural networks (NNs) in bioprocessing engineering nowadays. Neural networks can be very effective in predicting the performance of processes that are too complex and/or have only ill-defined models based on first principles. Combination of mass balances with the prediction capability of NNs may provide hybrid models able to capture relationships between different variables that affect enzyme kinetics—such as temperature and pH [21, 22]. Enzymatic depolymerization reactions, which inherently have complex substrates/products, have also been modeled by NNs [23]. In particular, multilayer perceptrons (MLPs) NNs [24, 25] have been successfully applied to solve this kind of problem.

A "neural-kinetic model" is proposed here, which expands the approach reported by Sousa et al. [26], to include the effects of temperature and pH on reaction rates. Peptides' molar mass distributions are inferred during the course of the reaction using MLPs. The product is lumped into five predefined ranges of molar mass. The neural-kinetic model presented here takes in account the influence of pH (between 8.5 and 10.4) and temperature (40–55 °C) on the action of alcalase® immobilized on glyoxyl-agarose gel particles, while hydrolyzing concentrated cheese whey.

Materials and Methods

Sweet cheese whey (*in natura*) was donated by Cooperativa de Lacticínios São Carlos (São Carlos, Brazil). Alcalase® 2.4 L (EC 3.4.21.62) was donated by Novo Nordisk do Brasil (Araucária, Brazil). It was multipoint-immobilized on 10% agarose beads (weight basis, particles with average diameter of 100 μm), activated with 210 μeq of aldehyde groups per milliliter of agarose support [9].

Concentration of cheese whey proteins, using micro- and ultrafiltration, was carried on using, respectively, a hollow fiber cross-flow system with a 0.36 m^2, 0.2 μm pore-size membrane (GE Biosciences, Piscataway, NJ, USA) and a Pellicon 2 tangential flow filtration module with 0.5 m^2 of a regenerated cellulose 10,000-Da-cutoff membrane (Millipore-Pellicon system,

Billerica, MA, USA). The micro- and ultrafiltration systems were run sequentially at 25 °C (gauge pressures up to 1 bar). After a 12-times reduction of the initial volume of whey (*in natura*), the process was interrupted, providing a concentration factor close to 10, that is, from 6.2 to 60.6 $g_{Protein}\ L^{-1}$. Concentrations of protein were measured by Kjeldahl method [27].

A Metrohm Titrino 718 pH-stat (Herisau, Switzerland) was employed for the kinetic assays at different values of pH and temperature (in batch operation mode). Alcalase® immobilized on glyoxyl-agarose gel [load of 2.8 *N*-benzoyl-L-arginine etyl ester (BAEE) units] was used in a suspension of 0.03 L containing 60 $g_{Protein}\ L_{Suspension}^{-1}$. One BAEE (from Sigma Chemical, St. Louis, MO, USA) unit (U_{BAEE}) was defined as the amount of enzyme that hydrolyses 1 μmol of BAEE per minute under standard conditions, as follows: 250 μL of immobilized enzyme suspension was added to the assay solution (2.5 mL of BAEE, 0.5 mM, prepared in 50 mM phosphate buffer, pH 7.6), and the increase in absorbance was measured during 5–10 min at 253 nm [28].

During each assay, free-of-enzyme samples containing 300 μL of hydrolyzed whey were taken periodically. These samples were analyzed using size exclusion chromatography through a Superdex Peptide HR 10/30 column (GE Biosciences). Mass concentrations of five ranges of predefined molar masses (MM) were determined: pseudocomponent 1, $MM_1 \leq 650$ Da; pseudocomponent 2, 650 Da$<MM_2 \leq$1,050 Da; pseudocomponent 3, 1,050 Da$<MM_3 \leq$4,150 Da; pseudocomponent 4, 4,150 Da$<MM_4 \leq$14,000 Da; pseudocomponent 5, 14,000 Da$<MM_5$. The calibration curves were fitted following the methodology described by Sousa et al. [26].

Experimental curves of pseudocomponents concentration vs. time were previously smoothed [29]. Reaction rates were obtained after the direct differentiation of these curves using the proper "calculus tool" of the software Microcal Origin (Microcal Software, Northampton, MA, USA). Figure 1 shows the evolution of a typical hydrolysis in a batch reactor. These chromatograms illustrate the complexity of the resulting mixture of peptides.

The vector of concentrations of the five predefined ranges of peptides' molar mass (our five pseudocomponents) maps into the vector of reaction rates of each pseudocomponent under the MLP network. The NNs were trained using the Neural Network Toolbox of Matlab 6.5 (MathWorks, Natick, MA, USA). The network learning function *TRAINLM*, available in this toolbox, employs the routine of Hagan and Menhaj, which combines the algorithms of Marquardt and back-propagation, in a batch supervised training [30]. Differential mass balances of the pseudocomponents were solved numerically, using the MLP output to predict reaction rates.

Figure 2 illustrates the neural-kinetic model: C_i and r_i are, respectively, mass concentration and reaction rates of pseudocomponent *i*. Table 1 presents the entire set of trained MLPs, which intended to cover all reasonable variations of pH and temperature around the optimum values for alcalase®-glyoxyl-agarose: pH=9.5 and T=50 °C [31]. The effects of pH and temperature were considered via linear interpolation. For example: if pH=9.3 and T=53 °C, the outputs of MLP-6 and MLP-7 (at T=55 °C) were interpolated. Then, the same procedure was done for MLP-2 and MLP-3 (at T=50 °C). Finally, these two sets of reaction rates were interpolated with respect to the temperature.

Mass balances for the batch, stirred reactor (assuming the volume of the system, V, to be constant) are:

$$\frac{dC_i}{dt} = Er_i, i = 1,5 \tag{1}$$

After the standard procedures of training and testing, the neural-kinetic model was incorporated to the process supervisory system for the real-time state inference of a

continuous bench-scale enzymatic reactor (0.5 L) with temperature control (thermostatic bath Brookfield EX200) and Masterflex L/S pumps for cheese whey feeding and product withdrawal. A GMC-Fuzzy control provided pH control, adding NaOH through a Prominent Gamma G/4b pump [31].

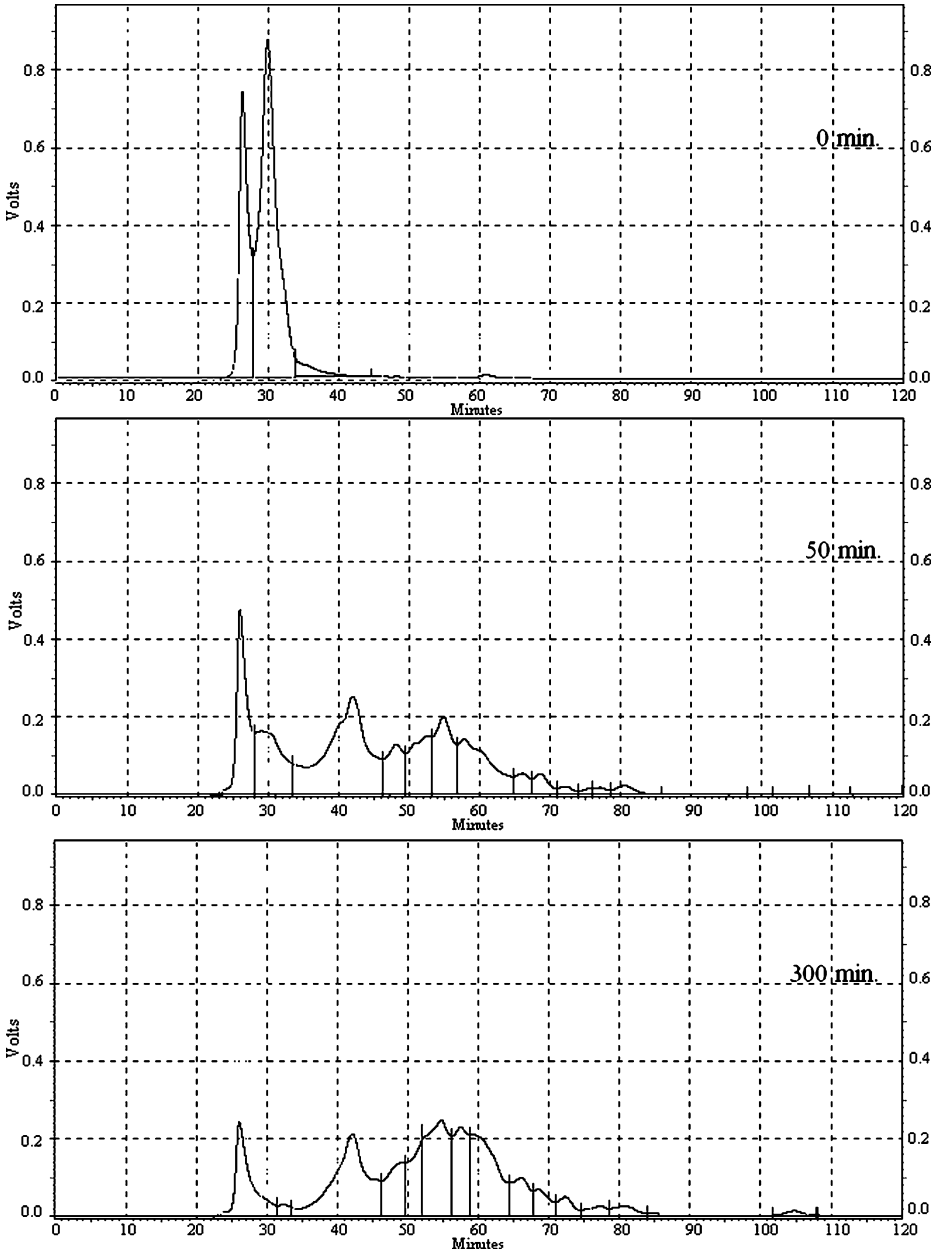


Fig. 1 Typical high-performance liquid chromatography profiles of alcalase® hydrolysates. Reaction run in a 0.03-L batch reactor; pH 8.5; T=40 °C; V=0.03 L; $C_5(0) = 53 g_{Protein}\, L_{Suspension}^{-1}$; $E = 134.4\ U_{BAEE}\, L_{Suspension}^{-1}$

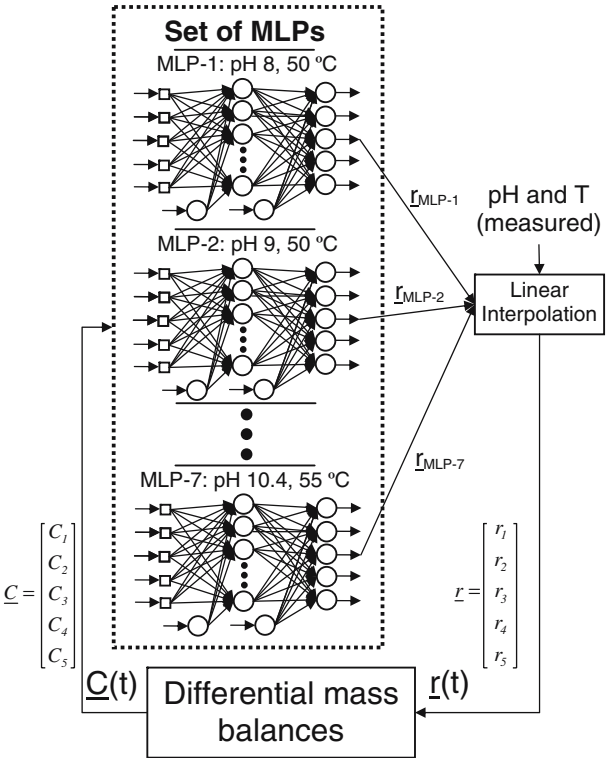


Fig. 2 Illustrative diagram of the neural-kinetic model of the hydrolysis of cheese whey

Results and Discussion

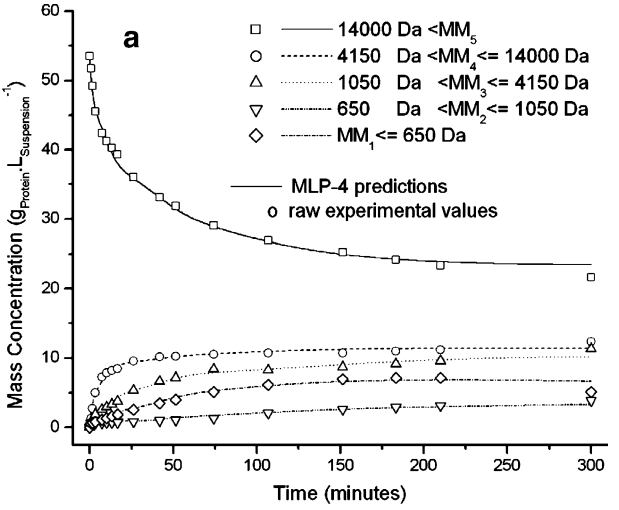
MLP Training and Test

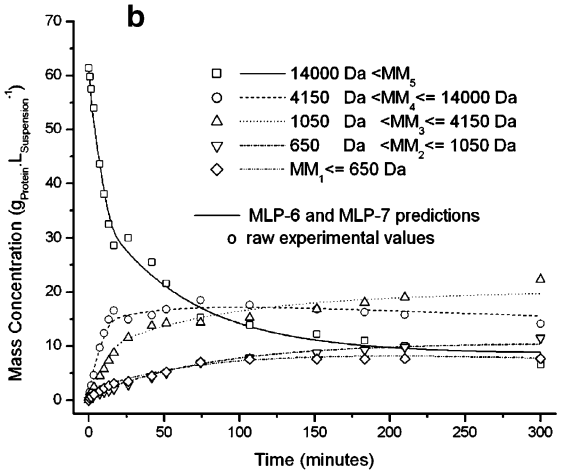
The MLP topology consisted of one hidden layer (nine neurons) and one output layer (five neurons). The activation function of both layers was a sigmoid. The use of a sigmoid function instead of a linear one in the output layer was necessary due to the nonlinear interdependency of the reaction rates. To avoid overfitting, the early-stopping technique was incorporated into the learning routine during the offline training procedure [25, 32].

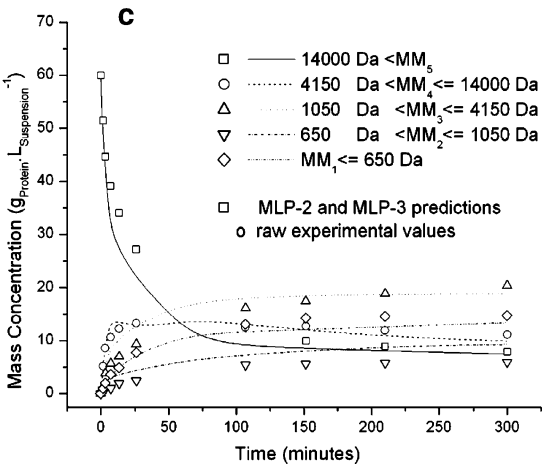
Figure 3a–c compares model results and raw experimental data (before smoothing) of three experimental data sets. Figure 3a shows the quality of fit of the hybrid kinetic model

Table 1 Set of trained MLPs.

MLP	pH	*T* (°C)
MLP 1	8.00	50.0
MLP 2	9.00	50.0
MLP 3	10.0	50.0
MLP 4	8.50	40.0
MLP 5	10.4	40.0
MLP 6	8.50	55.0
MLP 7	10.4	55.0


a
14000 Da <MM5
4150 Da <MM4<= 14000 Da
1050 Da <MM3<= 4150 Da
650 Da <MM2<= 1050 Da
MM1<= 650 Da
MLP-4 predictions
o raw experimental values
Mass Concentration (gProtein.LSuspension-1)
Time (minutes)


b
14000 Da <MM5
4150 Da <MM4<= 14000 Da
1050 Da <MM3<= 4150 Da
650 Da <MM2<= 1050 Da
MM1<= 650 Da
MLP-6 and MLP-7 predictions
o raw experimental values
Mass Concentration (gProtein.LSuspension-1)
Time (minutes)


c
14000 Da <MM5
4150 Da <MM4<= 14000 Da
1050 Da <MM3<= 4150 Da
650 Da <MM2<= 1050 Da
MM1<= 650 Da
MLP-2 and MLP-3 predictions
o raw experimental values
Mass Concentration (gProtein.LSuspension-1)
Time (minutes)

◀ **Fig. 3** Time course of the molar mass distribution of peptides (batch operation mode). **a** MLP-4, pH 8.5, T=40°C, $C_5(0) = 53 g_{Protein}\, L_{Suspension}^{-1}$. **b** Interpolation using MLPs 6 and 7 for pH 9.5; T=55 °C, $C_5(0) = 61 g_{Protein}\, L_{Suspension}^{-1}$. **c** Interpolation using MLPs 2 and 3 for pH 9.5; T=50 °C, $C_5(0) = 60 g_{Protein}\, L_{Suspension}^{-1}$. Other experimental conditions: V=0.03 L; $E = 134.4\ U_{BAEE}\, L_{Suspension}^{-1}$

(MLP-4) to a data set used for training. Experiments in Fig. 3b, c were not used to train the NNs, they were run at an intermediate pH, equal to 9.5. To calculate the concentrations at this pH, a linear interpolation was carried on between MLPs 6 and 7 (at 55 °C, Fig. 3b) and between MLPs 2 and 3 (at 50 °C, Fig. 3c). The evolution of the peptides' molar mass distribution was predicted by the model with remarkable accuracy. It should also be stressed that Hagan and Menhaj learning algorithm was very efficient in training MLPs 1–7, which have 104 weights each.

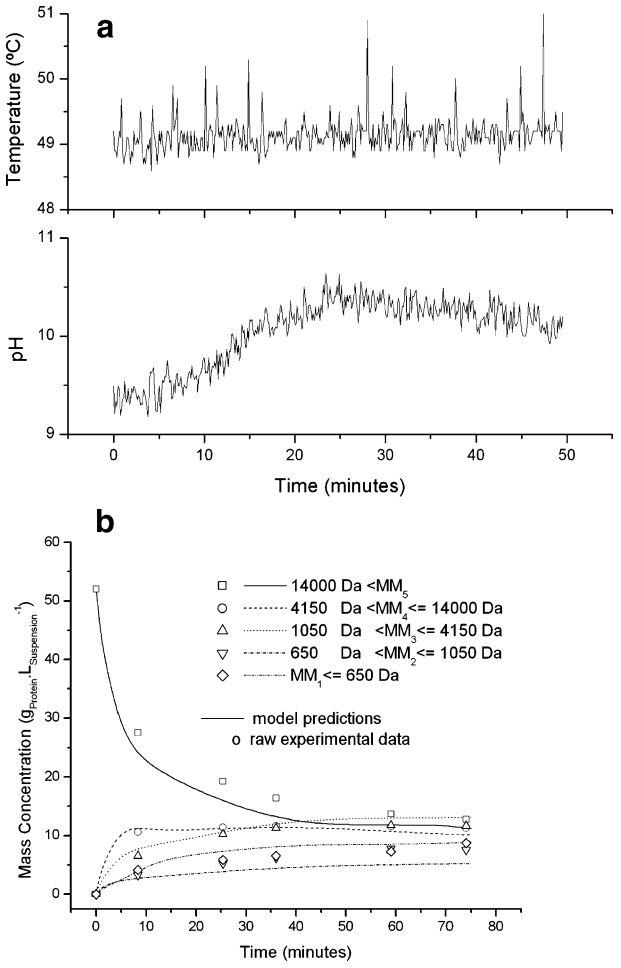


Fig. 4 Validation of the hybrid neural-kinetic model. **a** Online pH and temperature measurements—the magnitude of the random noise is apparent. **b** Distribution of peptides during the start-up of a continuous stirred tank reactor. Experimental nominal conditions: pH 9.5; T=50 °C; V=0.5 L; $C_5(0) = 52 g_{Protein} \cdot L_{Suspension}^{-1}$; $C_5^F = 52 g_{Protein} \cdot L_{Suspension}^{-1}$; q_F=0.1 L · h^{-1}; $E = 149.5\ U_{BAEE} \cdot L_{Suspension}^{-1}$

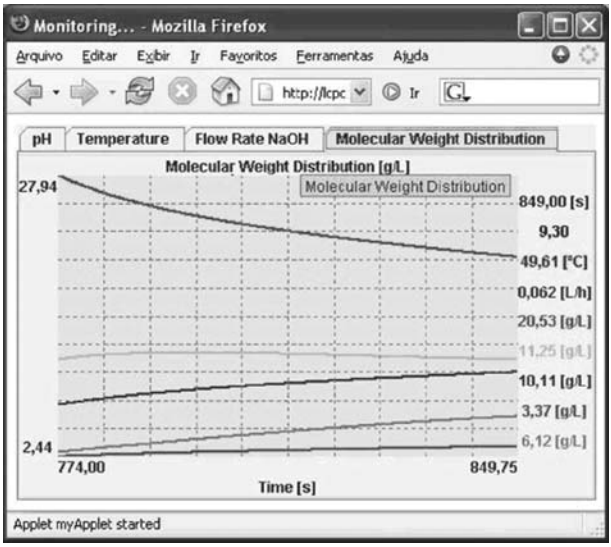


Fig. 5 Supervisory system graphical user interface: online remote monitoring of the proteolysis of cheese whey, showing the prediction of the concentration of five pseudocomponents (different range of peptides) after the start-up of a continuous stirred tank reactor

Implementation of the Model within the Supervisory System

The neural-kinetic model was incorporated to a supervisory program. Equations 2 and 3 make a set of ordinary differential equations (ODEs) that describe the pseudocomponent mass balances within the continuous, stirred enzymatic reactor:

$$\frac{dC_i}{dt} = E.r_i - \frac{q_F + q_C}{V} C_i, i = 1.4 \tag{2}$$

$$\frac{dC_5}{dt} = E.r_5 - \frac{q_F + q_C}{V} C_5 + \frac{q_F}{V} C_5^F \tag{3}$$

Figure 4b shows a strong validation test, where the neural-kinetic model was used to model the start-up of the continuous tank reactor at pH 9.5 and T=50 °C. Measurements of pH and temperature show white Gaussian noise (standard deviation, σ=0.35 for pH and σ= 0.26 °C), see Fig. 4a. The hybrid kinetic model results (MLPs 1–7) were linearly interpolated, providing predictions using the noisy online signals of pH and temperature, which were not filtered beforehand. The key idea in the present work was to interpolate the output of smaller MLPs, instead of trying to include pH and temperature in the input vector of a bigger NN. This approach turns easier the training of the NNs because it considerably reduces the complexity of the model. Besides, the proposed architecture for the state inference was, by itself, a test of the ability of the hybrid model to filter random noise. The smoothness of model predictions indicates that the association of the MLPs to a phenomenological model (the set of ODEs, Eqs. 2 and 3) was able to compensate the fast dynamics of the noise—a desirable property of MLP network systems [32].

Finally, Fig. 5 shows the graphical user interface of the remote supervisory system (programmed in Java, Sun Microsystems, Santa Clara, CA, USA), which provides an appealing and consistent description of the process, real-time updated during the hydrolysis of whey by alcalse®. With this system, online supervision of the evolution of peptides' molar mass distribution was successfully accomplished.

The methodology put forth here (including the training of the MLPs) can be implemented as a routine procedure, to be repeated when the characteristics of the substrate change along the year, due to seasonal changes in feedstock or in the upstream processing in the dairy industry. Following this procedure, lengthy offline high-performance liquid chromatography (size-exclusion) analyses would be necessary only during the training/test steps of new MLPs. Then, this new set of MLPs could be incorporated, for example, to the neural-kinetic model of an industrial supervisory system. We are presently working on the generalization of this procedure, using pattern recognition routines to classify the raw material and identify which set of MLPs should be used in each case.

Conclusions

A hybrid model of the enzymatic cheese whey proteolysis by alcalase®, multipoint-immobilized on glyoxyl-agarose gel particles was presented. A set of MLP NNs, coupled to differential mass balances, was used to predict the rate of disappearance of the primary substrate (whey), together with the rates of production of four pseudocomponents (representing pools of peptides within predefined ranges of molar masses). Seven MLPs were trained at fixed pH and temperatures (pH 8.5–10.5 and T=40–55 °C). Predictions of rates in pHs or temperatures not used for training the MLPs could be achieved using linear interpolation. The key idea was to interpolate the output of smaller MLPs, instead of increasing the dimension of the NN input vector to include pH and temperature. Thus, the complexity of the neural model was reduced, as well as the computational effort for training the NN.

The neural-kinetic model was incorporated as part of the monitoring system of a bench-scale continuous reactor and provided accurate predictions of the pseudocomponents profiles. The ability of the proposed hybrid model to filter noisy input signals was noticeable, which is an important feature for the control of the bioreactor. This methodology can be further applied to other reactions involving proteolysis of residues from different sources (for instance, the oil extraction industry).

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