IPC 2.0: prediction of isoelectric point and pKₐ dissociation constants

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ABSTRACT

The isoelectric point is the pH at which a particular molecule is electrically neutral due to the equilibrium of positive and negative charges. In proteins and peptides, this depends on the dissociation constant (pKₐ) of charged groups of seven amino acids and NH⁺ and COO⁻ groups at polypeptide termini. Information regarding isoelectric point and pKₐ is extensively used in two-dimensional gel electrophoresis (2D-PAGE), capillary isoelectric focusing (cIEF), crystallisation, and mass spectrometry. Therefore, there is a strong need for the in silico prediction of isoelectric point and pKₐ values. In this paper, I present Isoelectric Point Calculator 2.0 (IPC 2.0), a web server for the prediction of isoelectric points and pKₐ values using a mixture of deep learning and support vector regression models. The prediction accuracy (RMSD) of IPC 2.0 for proteins and peptides outperforms previous algorithms: 0.848 versus 0.868 and 0.222 versus 0.405, respectively. Moreover, the IPC 2.0 prediction of pKₐ using sequence information alone was better than the prediction from structure-based methods (0.576 versus 0.826) and a few folds faster. The IPC 2.0 webserver is freely available at www.ipc2-isoelectric-point.org

INTRODUCTION

The isoelectric point (pI or IEP) is an important physicochemical parameter of many compounds, including peptides and proteins, and can be used to estimate the surface charge of molecules in various pH conditions. This physicochemical property has been extensively used in many molecular techniques, such as two-dimensional gel electrophoresis (2D-PAGE) (1,2), capillary isoelectric focusing (3,4), crystallisation (5), and mass spectrometry (MS) (6,7). It should be stressed that for polypeptides, the isoelectric point depends mostly on the acid dissociation constants (pKₐ) of the ionisable groups of seven charged amino acids: glutamate (γ-carboxyl group), cysteine (thiol group), aspartate (ß-carboxyl group), tyrosine (phenol group), lysine (ε-ammonium group), histidine (imidazole side chains), and arginine (guanidinium group). Furthermore, other charged groups can be important, such as the amine and carboxyl-terminal groups of the polypeptide chain and the post-translational modifications (PTMs) that carry the charged groups (e.g. phosphorylation and N-terminal acetylation). Moreover, the difference between the theoretical pI and the
experimental pH can be related to the ionisation state of the individual residues. Some residues are buried inside the protein structure and, therefore, their contribution to the net charge of the whole molecule is marginal. Additionally, the charge of the exposed residue can be neutralised if it is used to form interactions with other residues, such as in non-covalent salt bridges, in which a proton migrates from a carboxylic acid group to a primary amine or to the guanidine group in Arg (in proteins, Lys or Arg are used as the bases and Asp or Glu as the acids; 8–10). However, most of the in silico methods that are currently used for pH estimation are based on simply counting the numbers of charged residues and utilising the Henderson-Hasselbach equation with customised pKa values (11,12). Nevertheless, some attempts to build more sophisticated methods should be acknowledged, such as those using genetic algorithms (13), artificial neural networks (14) and support vector machines (15).

While the estimation of the isoelectric point can be considered a challenging task, the prediction of pKa values for individual residues is even more difficult. The average pKa values used for pH prediction have been measured using simplified conditions, such as alanine pentapeptides with charged residue in the centre (16). This has been done to minimise the contribution from neighbouring residues, but such an approach is of no use for pKa estimation in real proteins, where the influence of surrounding residues must be considered. An additional problem involved in building a reliable pKa prediction algorithm is the scarcity of data (approximately 1000 known pKa values in proteins have been measured experimentally (17)). To date, for pKa prediction, only programs based on protein structure have been available, such as MCCCE (18), H++ (19), Propka (20) and Rosetta pKa (21).

In this work, I present a major update of the original IPC algorithm (available at http://isoelectric.org (12) that significantly extends its capabilities (Figure 1). The IPC 2.0 web server (available at http://www.ipc2-isoelectric-point.org) and mirrored at http://ipc2.mimuw.edu.pl) incorporates two major feature upgrades:

- Prediction of the isoelectric point using state-of-the-art machine learning instead of the relatively simple pKa optimisation used in IPC 1.0
- Prediction of individual pKa values based solely on sequence features

The features were implemented to address the major drawbacks of IPC according to users’ feedback and to improve prediction accuracy. Additionally, a new, larger dataset for peptides was used. The input of IPC 2.0 is the peptide or protein sequence(s) in a one-letter amino acid code (for multiple sequences, the FASTA format is used). For each input sequence, IPC 2.0 runs the machine learning models for the isoelectric point and predicts the pKa dissociation constant for each charged residue and the terminal groups of polypeptide chains. Additionally, a scatter plot with the predicted isoelectric points versus the molecular weight is presented for all proteins (in total, output from 21 isoelectric point prediction methods). All the prediction results can be downloaded in CSV format for further analysis.

### MATERIALS AND METHODS

#### Datasets

To develop and test the IPC2.0 server, multiple benchmark datasets had been used (Table 1). They can be divided into the following three main sets:

- **Protein dataset:** contains 2324 non-redundant proteins with experimentally measured isoelectric points (merged data from SWISS-2DPAGE and PIP-DB databases (22,23)). This dataset was also used in IPC 1.0. For more details, see (12).
- **Peptide dataset:** the datasets from previous studies were merged to form a total of 119,092 non-redundant peptides (6,24,25). Note that this is different peptide dataset than that used for IPC 1.0. For more details, see (12).
- **pKa dataset:** 1337 pKa values from 157 proteins were obtained from the PKAD database (17).

All datasets were clustered to avoid duplicates and, if needed, to merge and average experimental measurements. Next, the datasets were randomly split into 25% testing sets (used only for final benchmarks) and 75% training sets (used for machine learning, hyperparameters optimisation). All presented benchmarks on individual datasets had been calculated with 10-fold cross-validation.

#### Feature generation

The most important features for isoelectric point prediction are the sequence itself; the number of charged residues; the amino acid type on the C- and N-termini; and the isoelectric point predicted by simple methods using the Henderson–Hasselbach equation and pKa values, such as IPC (12), Bjellqvist (26) and DTASelect (27). To engineer additional features, the AAindex—with 566 matrices for 20 standard amino acids—was scanned (28). To select the most informative features, the univariate feature selection with regression (f_regression) and mutual information (mutual_info_regression) was used (up to 10 highest-scoring features were selected using the SelectKBest function from Scikit-learn) (29). For pKa models, the AAindex scores were calculated using kners of different sizes centred on a charged amino acid (for a pentamer, e.g. xxRxx, this may correspond to ALRWI, GIRAA, WRRIL, etc. For more details, see ‘Machine Learning Details’ section in the Supplementary Material). It is important to stress that local protein features, such as secondary structure and solvent accessibility, are valid only for protein sequences. They are irrelevant for short peptides, where, for instance, the use of a mass spectrometer disrupts any higher-order structure of the molecule. Therefore, pKa predictions should be considered valid only for proteins (> 50 amino acids).

#### Performance evaluation metrics

The prediction of pKa and pH values is a regression problem. Therefore, metrics such as root mean square deviation (RMSD), mean absolute error (MAE), Pearson’s correlation coefficient (r²), and the number of outliers were used. While the first three metrics are commonly used, the last must be explained. The outliers were defined at 0.5 and 0.25
Figure 1. Overview of the IPC 2.0 architecture. The input (amino acid sequence in the plain format or multiple sequences in the FASTA format) is processed by individual machine learning models. Separate models depending the prediction task are used. Isoelectric point prediction for peptides is based on separable convolution model (four channels representing the one-hot-encoded sequence, AAindex features, amino acid counts, and predictions from IPC 1.0). The protein pI and pK_a prediction models use the ensembles of low level models integrated with support vector regressor. For more details, see Supplementary Figure S1 and 'Machine Learning Details' in the Supplementary Material.

pH unit difference thresholds between the predicted and experimental pI for proteins and peptides, respectively. Thus, if the prediction disagree with the experimental pI by given threshold, such case has been considered as the outlier. The total number of outliers for individual datasets has been used to identify methods returning predictions within reasonable error range.

Machine learning
In this work, I try to solve three independent problems: the pI of proteins, the pI of peptides, and the pK_a of charged residues. Therefore, it is justified to design (at least) three separate models. Any machine learning process begins with the conversion of input data (in this case, polypeptide chains) into a format that can be used by machine learning packages (here, SciPy (30), sklearn (29), Tensorflow (31) and Keras (32)). The simplest approach is to use one-hot encoding, but it should be stressed that this produces a sparse matrix or vector (in this case, it would be $L \times 22$, where $L$ is the polypeptide length and 22 corresponds to 20 standard amino acid letters plus one for an unknown amino acid and one for padding). If the input varies in length, some padding (peptides; up to 60) or truncation (protein; down to 1000) is inevitable. With regard to pI prediction, the number and type of charged groups are most significant; thus, this information (even alone) can be used as the initial vector. Additionally, if possible, the introduction of hand-crafted fea-
The full datasets were never used directly. First, the sequences were clustered (to remove duplicates and to average isoelectric point if multiple experimental data existed), then split randomly into 25% and 75% sets (test and training data sets, respectively). The training sets were used for the training and (hyper)parameter optimisation. The test sets were used only once to assess the final performance of the models. For individual datasets’ sequences and experimental isoelectric points, see Supplementary Data 1.

In the case considered by this paper, apart from the sequence alone, I used features derived from the sequence (such as charge, length, molecular weight, hydrophybicity, number of charged amino acids, and the predicted pI from other methods). The input differs according to the problem to be solved and the machine learning technique used but, in general, the input consists of two major parts: sequence-related and feature-related. Several machine learning approaches were tested. First, I used optimization techniques to find the optimal set of seven pKa values for charged residues. In the first version of IPC, basin-hopping with a truncated Newton algorithm (33) was used; here, I used a differential evolution algorithm (34), as it performed significantly better. The population size was set to 50 and all remaining parameters were default (SciPy version (30)). Next, having the initial predictions of pI from IPC 1.0, I designed a very simple approach based on support vector regression (SVR) with RBF kernel and GridSearchCV parameters optimization. The input vector in this case was 19 predicted isoelectric points. Finally, I progressed to more advanced machine learning techniques, namely, deep learning. It is possible to start from simple dense networks (Multi-Layer Perceptron; MLP) with different numbers of dense layers and neurons that are interconnected with dropout and with different activation layers (preferably selu and elu). The final architecture for peptide isoelectric point prediction benefit from all mentioned information and is based on stacking of separable convolution layers. The input is reshaped in the following way. For peptides, the maximal length is 60 amino acids; thus, this defines the main size of the 2D ma-

tribox obtainable after one-hot encoding (all sequences were padded up to 60, if needed). This results in a matrix of 60 × 22 (20 standard amino acids, X for unknown, and 0 for padding). By analogy with the image processing from which convolution has been adopted, this 60 × 22 matrix can be considered as the main image size. Similarly, additional information can be stored in separate channels (for instance an RGB image has three channels for values of red, green, and blue). Here, I used four channels. The first channel was, as stated, a one-hot encoded sequence. The second channel stored information about the most informative features from AAindex. In the third and fourth channels, I encoded the information about 1D features, the information about charged residues counts, and the isoelectric point prediction from IPC 1.0. The input was processed by two separable convolution layers, interconnected with two average pooling layers. The initial kernel size of the filter was set to 22 × 5 to slide across the whole amino acid frame with a window of five amino acids. Then the feature maps were flattened and sent to a standard MLP unit: three dense layers (Supplementary Figure S1).

Machine learning architecture for the prediction of pKa is very different, as here the focus is on a single charged amino acid (and its neighbourhood). Therefore, the input is very limited. I decided to use the information related to kmers of different size. With increasing size of the kmer (from three to 15), we encoded the sequence (one-hot encoding) and the amino acid scores for the most informative features from AAindex. This information was used as input for the MLP unit (three dense layers separated by dropout layer). Next, to boost the performance we used an ensemble of nine models to build final support vector regression model.

In all deep learning models, the ADAM optimiser (35) and hyper-parameter optimisation by RandomizedSearchCV were used. As the optimisation condition, the mean_squared_error loss function was used. Apart from the dropout, 10-fold cross-validation and early stopping were used to estimate the robustness of the predictions and to
avoid overfitting. During the fitting process, the training set was randomly split (validation_split = 0.2). Finally, the performance was estimated for the 25% of cases that had been omitted. For more details, see ‘Machine Learning Details’ in the Supplementary Material.

Other methods

To benchmark IPC 2.0, multiple other methods were compared. The simplest methods of isoelectric point prediction are based on different $pK_a$ sets and the Henderson–Hasselbach equation (Patrickios (36), Solomon (37), Lehninger (38), EMBOSS (39), Dawson (40), Wikipedia ($pK_a$ values as presented in Wikipedia page in 2005), Toseland (41), Silero (42), Thuirkvist (16), Rodwell (43), Wikipedia, (44), Grimsley (45), Bjellqvist (26), whose predictions were compared to those of Rosetta $pK_a$ (four variants) (21).

Implementation

The pre-processing, training, and testing of the machine learning models were done in the Python programming language. Among the libraries used, the most important were SciPy (30), sklearn (29), TensorFlow (31), and Keras (32). For the web-server implementation, the Apache server and the PHP programming language were used. In addition, the HTML front-end benefitted from the Twitter Bootstrap and CanvasJS libraries.

RESULTS

Isoelectric point prediction

The isoelectric point prediction of the IPC 2.0 method is based on two separate datasets that consist of protein and peptides. The datasets differ in size and in the difficulty of the prediction task. Peptides are much shorter and contain only a small number of charged groups. In contrast, in proteins, multiple additional factors, such as PTMs or solvent accessibility, need to be taken into account. Thus, the estimation of the isoelectric point is much more difficult. The results presented in Table 2 show that the IPC 2.0 models performed the best. Moreover, the more information and more advanced machine learning technique used, the better were the results. The optimisation models (denoted IPC2.protein and IPC2.peptide) use $pK_a$ sets that are optimal for calculating $pI$ with the Henderson–Hasselbach equation (Supplementary Table S1). They perform better than any other methods (RMSE of 0.860 by IPC2.protein versus 0.911 by ProMoST and 0.248 by IPC2.peptide versus 0.405 by Bjellqvist). However, from an machine learning point of view, they are very simple (but at the same time very fast).

The next machine learning approach I used was support vector regression (SVR), in which the isoelectric point prediction accuracy on leave-out 25% datasets

| Method                             | RMSE | MAE   | $R^2$ | Outliers |
|------------------------------------|------|-------|-------|----------|
| IPC2.protein.svr.19                | 0.8479 | 0.5906  | 0.5934  | 247      |
| IPC2.protein                      | 0.8608 | 0.6052  | 0.5748  | 251      |
| IPC2.protein                      | 0.8677 | 0.6109  | 0.5760  | 250      |
| ProMoST                            | 0.9113 | 0.6444  | 0.5183  | 263      |
| Toseland                           | 0.9278 | 0.6537  | 0.5095  | 250      |
| Dawson                             | 0.9365 | 0.6586  | 0.4977  | 263      |
| Bjellqvist                         | 0.9369 | 0.6536  | 0.5005  | 260      |
| Wikipedia                          | 0.9484 | 0.6795  | 0.4860  | 262      |
| Rodwell                            | 0.9579 | 0.6762  | 0.4706  | 262      |
| Grimsley                           | 0.9588 | 0.6953  | 0.4779  | 265      |
| Lehninger                          | 0.9617 | 0.6783  | 0.4607  | 266      |
| Solomon                            | 0.9631 | 0.6746  | 0.4606  | 272      |
| pIR                                | 1.0148 | 0.7556  | 0.4161  | 315      |
| Nozaki                             | 1.0164 | 0.7219  | 0.3980  | 288      |
| Thuirkvist                         | 1.0250 | 0.7573  | 0.3948  | 302      |
| DTASelect                          | 1.0278 | 0.7798  | 0.3947  | 319      |
| EMBOSS                             | 1.0498 | 0.7757  | 0.3734  | 308      |
| Silero                             | 1.0519 | 0.7694  | 0.3461  | 308      |
| Patrickios                         | 2.3764 | 1.8414  | <0      | 517      |
| Predp1-TMT6                        | NA    | NA     | NA     | NA       |
| Predp1-plain                       | NA    | NA     | NA     | NA       |
| Predp1-iTRAQ8                      | 0.4235 | 0.2796  | 0.9103  | 10606    |
| Predp1-TMT6                        | 0.6365 | 0.3518  | 0.7988  | 12135    |
| Predp1-plain                       | 0.6480 | 0.3710  | 0.7913  | 12813    |
| Patrickios                         | 2.3764 | 1.8414  | <0      | 517      |
| Predp1-TMT6                        | NA    | NA     | NA     | NA       |

a values as presented in Wikipedia page in 2005), Toseland (41), Silero (42), Thuirkvist (16), Rodwell (43), Wikipedia, (44), Grimsley (45), Bjellqvist (26), whose method was implemented as ExPASy ‘Compute pI/Mw Tool’ (46), ProMoST (9) and finally IPC 1.0 (12). Additionally, machine learning methods, such as Predp1 (plain, TMT6, iTRAQ8 variants) (6) and pIR (15) were also used. Furthermore, IPC 2.0 $pK_a$ predictions were compared to those of Rosetta $pK_a$ (four variants) (21).
dicted by other methods was an input, in a so-called ensemble averaging technique (47,48). The main advantage of SVR is that it has only two parameters (C and gamma for RBF kernel) that need to be optimised. The main disadvantage is that the input features must be already well designed and in a similar space (for instance, enriching the input with more heterogeneous features, such as protein length or molecular weight, prevent the algorithm from converging). The SVR models not surprisingly performed better than the optimisation versions (RMSE of 0.848 and 0.230 for proteins and peptides, respectively). Another key feature of ensemble averaging is that it can be used to reduce the variance of the predictions. This can be seen in this study with the significant reduction of outliers: the IPC2.peptide.svr.19 ensemble averaging is that it can be used to reduce the variance of the predictions. This can be seen in this study with the significant reduction of outliers: the IPC2.peptide.svr.19 ensemble averaging is that it can be used to reduce the variance of the predictions. This can be seen in this study with the significant reduction of outliers: the IPC2.peptide.svr.19 ensemble averaging is that it can be used to reduce the variance of the predictions. This can be seen in this study with the significant reduction of outliers: the IPC2.peptide.svr.19 ensemble average of nine ML models that use the information derived from k-mers of different sizes centred on the charged residue. This approach made it possible to capture sequence fingerprints that were located in direct proximity to the charged residue. The overall accuracy of the IPC 2.0 pKa prediction was better than that of the pKa Rosetta protocol used for benchmarking in this work requires several hours for a single protein). In contrast, IPC 2.0 uses only sequence-based features and returns results almost instantly with similar accuracy (Table 3 and Supplementary Table S7). The prediction of pKa values is based on an SVR ensemble of nine MLP models that use the information derived from k-mers of different sizes centred on the charged residue. This approach made it possible to capture sequence fingerprints that were located in direct proximity to the charged residue. The overall accuracy of the IPC 2.0 pKa prediction was better than that of the pKa Rosetta protocol (0.576 versus 0.839), although some pKa dissociation constants were clearly worse predicted by IPC 2.0. The main sources of misprediction were His and Tyr residues: for Tyr residues at least, this can be explained by the small number of training points.

**pKₐ dissociation constant prediction**

The pKₐ prediction of individual residues is a separate challenge with its own problems that need to be addressed. First, the data are very limited. Moreover, currently available methods (e.g. MCCE, H++ and pKₐ Rosetta) require protein structure and are relatively slow (for instance, the pKₐ Rosetta protocol used for benchmarking in this work requires several hours for a single protein). In contrast, IPC 2.0 uses only sequence-based features and returns results almost instantly with similar accuracy (Table 3 and Supplementary Table S7). The prediction of pKₐ values is based on an SVR ensemble of nine MLP models that use the information derived from k-mers of different sizes centred on the charged residue. This approach made it possible to capture sequence fingerprints that were located in direct proximity to the charged residue. The overall accuracy of the IPC 2.0 pKₐ prediction was better than that of the pKₐ Rosetta protocol (0.576 versus 0.839), although some pKₐ dissociation constants were clearly worse predicted by IPC 2.0. The main sources of misprediction were His and Tyr residues: for Tyr residues at least, this can be explained by the small number of training points.

**IPC 2.0 web server**

The IPC 2.0 web server (http://www.ipc2-isoelectric-point.org) takes protein(s) and peptide(s) as input (single sequence or multiple sequences in FASTA format, up to 10 000 residues in total) and returns predictions of pI and pKₐ values for individual charged residues. In addition, the outputs are complemented by virtual 2D-PAGE plots. As the server is capable of multiple sequence predictions, its convenient output feature is the CSV format, which can be used for further large-scale analyses (e.g. on the proteome scale 49–52). Additionally, the standalone version of IPC
2.0 is also available on web server site and as Supplementary Data.

DISCUSSION

In this work, I have presented IPC 2.0, a new web server for isoelectric point and pKa dissociation constant prediction based on sequence information only. It uses a state-of-the-art ML methodology and represents an improvement on previous methods. Additionally, IPC 2.0 is the first, fast pKa prediction method that can estimate pKa values using sequence information alone. It does so within seconds, with a performance level similar to more time-consuming and structure-based methods. To boost the performance of IPC 2.0 and to bypass the limited size of datasets for protein and pKa prediction hand-crafted features were used (Supplementary Tables S6 and S7).

However, users should be aware of some of the IPC 2.0 web server’s limitations. First, the isoelectric point and pKa predictions can be significantly distorted when PTMs are present (e.g. phosphorylation). This should be kept in mind when analysing proteins from eukaryotes that are rich in PTMs (see Supplementary Table S1 in Kozlowski, 2016 [12]). Due to very limited experimental data, it was not possible to develop machine learning models dedicated to PTMs. Another shortcoming of IPC 2.0 is that it only uses sequence information. It is expected that protein structures that contain more information about the charged residue’s neighbourhood should improve the prediction performance. Such an approach could help to increase the modest performance of pKa predictions.

The high performance of the IPC 2.0 web server (and standalone version) makes it suitable for large-scale analyses related to the charge of proteins and peptides. I believe that biologists will benefit from this web server with its user-friendly interface.

DATA AVAILABILITY

IPC 2.0 (http://www.ipc2-isoelectric-point.org), including the web service, datasets, standalone scripts, and documentation, has been donated to the public domain. Therefore, it can be freely used for any legal purpose. Nevertheless, the machine learning libraries used by some models are: sklear, Tensorflow and Keras, which are under BSD, Apache and MIT licences, respectively. The web server will be available at the given web address for at least 10 years.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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