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First Bovine Serum Albumin-Promoted Synthesis of Enones, Cinnamic Acids and Coumarins in Ionic Liquid: An Insight into the Role of Protein Impurities in Porcine Pancreas Lipase for Olefinic Bond Formation

Nandini Sharma,[a] Upendra K. Sharma,[a] Rajesh Kumar,[a] Nidhi Katoch,[a] Rakesh Kumar,[a] and Arun K. Sinha[a,*]

[a] Natural Plant Products Division, Institute of Himalayan Bioresource Technology, Council of Scientific and Industrial Research (CSIR), Post Box No. 6, Palampur – 176061 HP, India
Fax: (+91)-1894-230433; e-mail: aksinha08@rediffmail.com

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Abstract: During studies on exploiting the catalytic promiscuity of crude porcine pancreas lipase (PPL) in ionic liquid for C=C bond formations, bovine serum albumin (BSA) was found to be competing for these reactions. After a detailed investigation, we establish that these transformations are possible by unspecific protein catalysis rather than catalytic promiscuity of "PPL" – a first insight into the role of protein impurities in crude enzyme. Thus, a novel and highly efficient, environmentally friendly approach involving synergistic catalysis by bovine serum albumin-1-butyl-3-methylimidazolium bromide (BSA-[bmim]Br) has been developed for the synthesis of (*E*)-α,β-unsaturated compounds including a one-pot cascade synthesis of cinnamic acids and coumarins *via* aldol, Knoevenagel and Knoevenagel–Doebner condensations.

Keywords: aldol condensation; bovine serum albumin; ionic liquids; Knoevenagel condensation; Knoevenagel–Doebner condensation

The formation of the olefinic (C=C) bond, a fundamental transformation in organic synthesis, is well represented by aldol and Knoevenagel condensations. This transformation is generally achieved in the presence of a strong acid/base[1] or metal ions as catalyst;[2] thus raising serious environmental concerns. Recently some alternative approaches[3] using organic and heterogeneous catalysts for the aldol condensation have been reported; still the development of new cross-coupling methods beneficial in terms of environmental sustainability, operational simplicity, broad substrate scope, selectivity and involving novel cascade strategies is of significant concern.

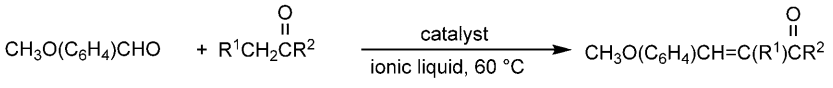
Since the past decade, dynamic efforts to exploit the biological potential of enzymes for catalyzing organic reactions is on the rise due to their simple processing requirements and high selectivity.[4] Among all enzymes, lipases top the list due to their remarkable versatility for the range of substrates and biotransformations they catalyze.[5] Recently, a new frontier, termed as biocatalytic promiscuity, has attracted much attention and several lipase-catalyzed C–C bond forming reactions[6] have been developed.

On the other hand, for C=C bond formations, very few enzymatic systems are reported. However, these procedures are hampered by the use of organic solvents or additives,[6c] lower product yields, prolonged reaction times and limited substrate scope. Accordingly, the development of new biocatalytic procedures might help to improve the existing catalytic conditions.

In addition, due to environmental concerns, "green solvents" are becoming an ideal medium for organic reactions. One such group of promising green solvents is "ionic liquids (IL)" because of their unique properties of non-volatility, non-flammability, recyclability, and ability to dissolve a wide range of materials.[7] In our previous study, we have observed promiscuous behaviour of *Candida antarctica* lipase in IL as H_2O_2 activator for alcohol oxidation under neutral conditions.[8] This case and other relevant reports encouraged us to explore the catalytic promiscuity of lipase for olefinic bond formation in IL.

Initially, we screened different commercially available lipases for the condensation of 0.12 mmol of 4-

Table 1. Optimization study of aldol condensation of 4-methoxybenzaldehyde with different ketones.[a]



Entry	Catalyst	Ketone R^1	Ketone R^2	Ionic liquid	Product [%][b]
1	PPL	H	CH_3	[bmim]Br	83
2	CAL-B	H	CH_3	[bmim]Br	nc
3	CCL	H	CH_3	[bmim]Br	4
4	CRL	H	CH_3	[bmim]Br	3
5	PCL	H	CH_3	[bmim]Br	nc
6	MJL	H	CH_3	[bmim]Br	10
7	TLL	H	CH_3	[bmim]Br	6
8	PPL	H	CH_3	[bmim]Cl	37
9	PPL	H	CH_3	[bmim]BF_4	2
10	PPL	H	CH_3	[hmim]Br	44
11	PPL	H	CH_3	[bmim]OH	24
12	PPL	H	CH_3	[bmim]PF_6	29
13	PPL	H	CH_3	[Hmim]*p*TSA	5
14	PPL	H	CH_3	-	2
15	-	H	CH_3	[bmim]Br	nc
16	PPL	H	CH_3	MIm	traces
17	PPL	H	C_2H_5	[bmim]Br	51 (73)[d]
18	PPL	H	$CH_2CH(CH_3)_2$	[bmim]Br	58 (75)[d]
19	PPL	C_6H_5CO	CH_3	[bmim]Br	5 (20)[d]
20	PPL	Cl	CH_3	[bmim]Br	23 (51)[d]
21	PPL	cyclohexanone		[bmim]Br	22 (56)[d]
22	PPL	cyclopentanone		[bmim]Br	36(63)[d]
23	PPL	H	C_6H_5	[bmim]Br	nc
24	BSA	H	CH_3	[bmim]Br	89
25	BSA	H	CH_3	-	traces
26	denatured PPL[c]	H	CH_3	[bmim]Br	34
27	lysine	H	CH_3	[bmim]Br	72
28	egg albumin	H	CH_3	[bmim]Br	71

[a] *Reaction conditions:* 0.12 mmol **1a**, 50 mg catalyst, 0.5 mL IL, 0.5 mL ketone, 6 h. CAL-B = *Candida antarctica* lipase B; CCL = *C. cylindracea* lipase; CRL = *C. rugosa* lipase; PPL = porcine pancreas lipase; PCL = *Pseudomonas cepacia* lipase; MJL = *Mucor javanicus* lipase; TLL = *Thermomyces lanuginosus* lipase.
[b] Yield on the basis of HPLC against reference standard.
[c] Pre-treated with urea and thiourea (7:1) at 100 °C for 2 h.
[d] Conversion after 48 h; nc = no conversion.

methoxybenzaldehyde (**1a**) with acetone using 1-butyl-3-methylimidazolium bromide ([bmim]Br) as a representative IL. Under the optimized conditions (see Supporting Information, Table SI), "PPL" provided best results (83% yield) with all other lipases failing to provide any significant yield of 4-methoxyphenylprop-2-en-1-one (**1b**) (Table 1, entries 1–7). Replacement of [bmim]Br with other ILs provided **1b** in low yield (Table 1, entries 8–13).

To exclude any background activity, control experiments carried out in the absence of IL (Table 1, entry 14) or lipase (Table 1, entry 15) provided **1b** only in traces even after 72 h. Similarly, a reaction carried out with 1-methylimidazole (MIm) (precursor for [bmim]Br) provide a trace yield; ruling out catalytic activity from residual MIm (Table 1, entry 16), if any. Further experiments carried out for the condensation of **1a** with different ketones (Table 1, en-

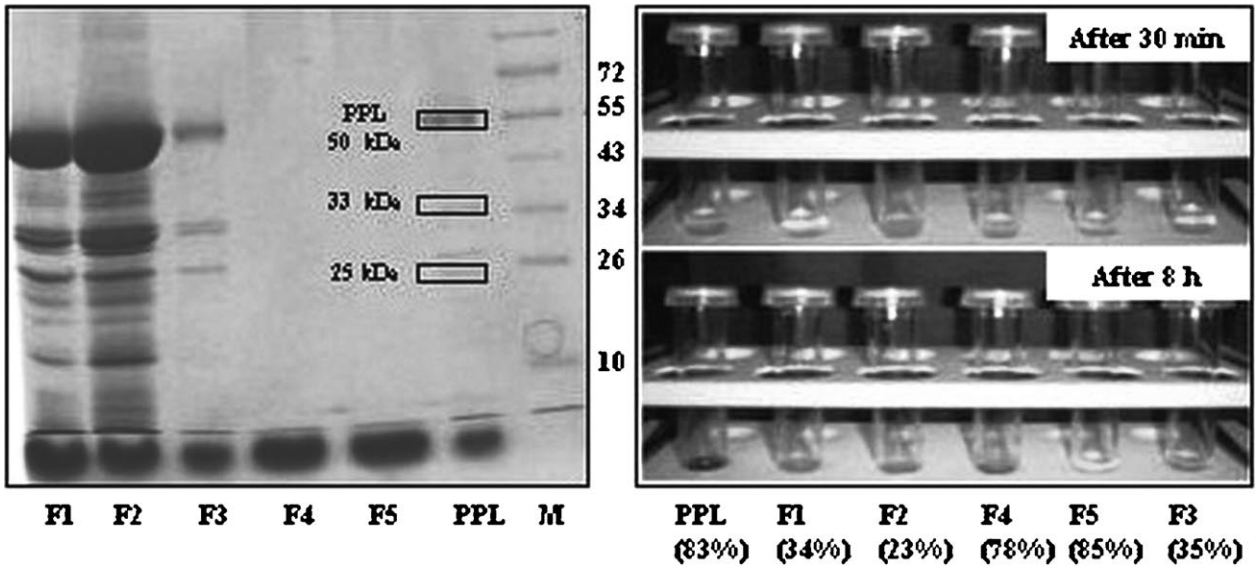


Figure 1. SDS page analysis of different fractions prepared from PPL and photographs of reaction mixture at different time intervals; F1, F2, F3, F4, F5 as mentioned in Scheme S1 (see Supporting Information), M = marker.

tries 17–23) indicated that the above reaction prefers acetone over other ketones.

Amazingly, replacement of "PPL" with "off-the-shelf'' protein that is, bovine serum albumin, resulted in 89% yield (Table 1, entry 24) although BSA alone provided a trace yield (Table 1, entry 25). In addition, a moderate yield was also obtained with denatured lipase (Table 1, entry 26). These anomalous results (in comparison to a previous report by Li et al.[6f]) together with the crude nature of "PPL" made us sceptical about the involvement of the enzyme in the reaction. There are reports that crude preparations of "PPL", apart from true lipase (protein band at 50 kDa), contain a significant number of other proteins as impurities.[9] Thus, we decided to enrich "true PPL" by fractionation and to explore its exact role as catalyst. Accordingly, five different fractions from the crude enzyme were prepared and tested for the aldol condensation. Interestingly, fractions F4 and F5 containing peptides and residual amino acids of molecular weight less than 10 kDa provided best results while the "PPL"-containing fractions (F1 and F2) gave significantly lower yields; suggesting the role of protein catalysis in the above reaction (Figure 1).

To further validate our hypothesis of protein catalysis and the nature of catalysis (specific or unspecific), we performed experiments using BSA, lysine and egg albumin as catalyst under the optimized conditions. Almost similar yields were observed in all cases (Table 1, entries 27 and 28) confirming the role of non-specific protein catalysis for the aldol condensation. Since the completion of this work, a protein-catalyzed Henry reaction[10] was reported which corroborated our findings.

To investigate the catalytic potential of BSA-[bmim]Br under the optimized conditions for aldol condensation, we studied the substrate scope for wide range of aldehydes (Table 2).

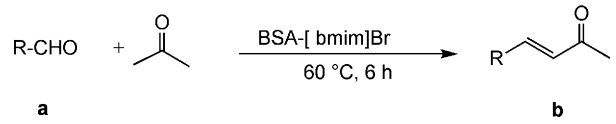
Good to excellent product yields were observed in all cases. In particular, the catalyst exhibited remarkable activity even for halogen-, nitrogen- or *N*,*N*-dimethyl-containing compounds ensuing the exclusive formation of the enone product[11] (Table 2, entries 9, 17, 19, 20). In addition, the above biocatalytic approach provided a facile access towards the synthesis of hydroxy-substituted enones (Table 2, entries 4–7) that otherwise requires protection-deprotection strategies.[12]

In order to take the advantage of the developed approach, we successfully extended it towards citral for the synthesis of *E*-pseudoionone,[13] a key starting material for the synthesis of vitamin A and carotenoids etc. (Scheme 1).

The next important aspect was examination of the possibility for catalyst reuse in subsequent reactions (examined for up to four cycles). The recovered catalyst showed complete conversion for first two cycles and a slight decrease in activity was recorded for the third and fourth cycles (80% conversion) which was compensated by adding few mg of fresh BSA.

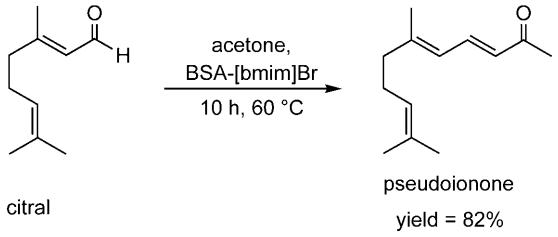
To obtain more insight into the catalytic possibilities of BSA, we investigated the product formation for a series of aldehydes with active methylene groups. This reaction, known as the Knoevenagel condensation, occurs in the presence of acid/base catalyst or a heterogeneous support.[14] Of late, the use of guanidine-based task-specific ionic liquids,[15] and amino acids as a promoter in ionic liquids[16] has also been established.

Table 2. BSA-catalyzed aldol condensation in [bmim]Br.[a]



Entry	R	Product [%][b]	Entry	R	Product [%][b]
1	4-$MeOC_6H_4$	89 (86)[c]	11	2-OAc-3-$MeOC_6H_3$	52 (46)[c]
2	2,4-$(MeO)_2C_6H_3$	83	12	2-C_4H_3O	90
3	2,4,5-$(MeO)_3C_6H_2$	80 (74)[c]	13	1-$C_{10}H_7$	68
4	4-OH-3-$MeOC_6H_3$	90 (87)[c]	14	biphenyl	72[d]
5	4-OHC_6H_4	88 (84)[c]	15	3-C_8H_6N	57[d]
6	2-OH-3-$MeOC_6H_3$	85 (81)[c]	16	4-MeC_6H_4	94 (89)[c]
7	3,4-$(OH)_2C_6H_3$	92	17	3-BrC_6H_4	93
8	5-allyl-3,4-$(MeO)_2C_6H_2$	84 (79)[c]	18	3-$MeOC_6H_4$	89 (87)[c]
9	4-$N(Me)_2C_6H_4$	90 (85)[c]	19	4-ClC_6H_4	93
10	4-$MeOC_6H_4CH{=}CH$	nc	20	4-$NO_2C_6H_4$	77[d]
			21	2-C_4H_3S	87

[a] *Reaction conditions:* 0.12 mmol substrate, 50 mg BSA, 0.5 mL IL, 0.5 mL acetone.
[b] On HPLC basis and product confirmation by ESI-MS/MS and NMR (with *E*-selectivity).
[c] Isolated yield in parentheses.
[d] In 72 h.



Scheme 1. BSA-IL-catalyzed synthesis of pseudoionone.

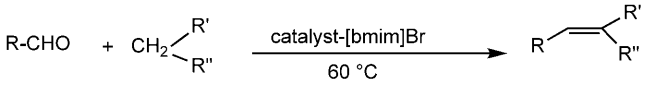
As expected, quantitative conversion was observed with BSA, PPL as well as lysine for the condensation of 4-methoxybenzaldehyde (**1a**) and diethyl malonate under optimized conditions (Table 3, entries 1–3). strengthening our perception of protein catalysis rather than lipase promiscuity. These results were in contrast to a recent report by Lai et al.[17] where the possibility of protein catalysis has been ruled out. To substantiate our findings, we decided to perform two set of experiments with BSA at 60 °C employing our optimized conditions (in [bmim]Br) and the conditions reported by Lai et al. (in DMSO).[17] Surprisingly, we observed significant conversion (up to 70%) in both cases. Thereafter, we studied the scope of the BSA-catalyzed Knoevenagel reaction towards various active methylene groups. As evident from Table 3, condensation of aromatic aldehydes with malononitrile occurred at a very fast rate (within 1 h) providing excellent yields. Reactions with ethyl acetoacetate and ethyl cyanoacetate took 4 to 8 h for conversion providing products with *(E)* selectivity.

Interestingly, the condensation of malonic acid with aldehydes was followed by decarboxylation (a case of Knoevenagel–Doebner condensation); furnishing the corresponding cinnamic acids in high yields (Table 4).

Thus, the developed approach offers a remarkable improvement over classical conditions and results in an efficient, mild and base-free synthesis of several substituted cinnamic acids with high selectivity.

Another, important utility of the BSA-catalyzed approach surfaced in the case of reactions of *o*-hydroxy substituted benzaldehydes with malonic acid, diethyl malonate and ethyl acetoacetate. Here the condensation was accompanied by decarboxylation and subsequently cyclization furnishing coumarins in good yields (Table 5). Coumarins constitute one of the biologically important class of heterocyclic compounds and numerous methodologies have been reported for their synthesis; however, these generally involve harsh conditions (acidic or basic), multiple-step synthesis, lengthy work-up procedures and sometimes elevated temperatures or specialized instruments.[18] Thus, the present study provides a new perspective for the direct synthesis of cinnamic acids and coumarins (Knoevenagel–Doebner condensations) under mild and neutral reaction conditions.

Table 3. BSA mediated Knoevenagel condensation in [bmim]Br.[a]

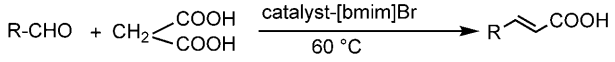


a b

Entry	R	R'	R''	Catalyst	Time [h]	Product [%][b]
1	4-$MeOC_6H_4$	COOEt	COOEt	BSA	8	72
2	4-$MeOC_6H_4$	COOEt	COOEt	PPL	8	71
3	4-$MeOC_6H_4$	COOEt	COOEt	lysine	8	66
4	4-$MeOC_6H_4$	CN	CN	BSA	1	91 (89)[c]
5	4-$MeOC_6H_4$	COOEt	$COCH_3$	BSA	8	73 (68)[c]
6	4-$MeOC_6H_4$	COOEt	CN	BSA	4	94 (87)[c]
7	C_6H_5	COOEt	CN	BSA	4	93
8	4-OHC_6H_4	CN	CN	BSA	0.5	90
9	4-OHC_6H_4	COOEt	COOEt	BSA	8	69
10	4-OH-3-$MeOC_6H_3$	CN	CN	BSA	0.5	86 (84)[c]
11	4-OH-3-$MeOC_6H_3$	COOEt	COOEt	BSA	8	74
12	4-ClC_6H_4	CN	CN	BSA	0.5	95
11	4-ClC_6H_4	COOEt	COOEt	BSA	8	94
12	4-$NO_2C_6H_4$	CN	CN	BSA	1	94 (86)[c]
13	4-$NO_2C_6H_4$	COOEt	COOEt	BSA	4	40
14	2-C_4H_3O	CN	CN	BSA	1	65
15	2-C_4H_3O	COOEt	COOEt	BSA	4	95 (84)[c]

[a] *Reaction conditions:* 0.12 mmol substrate, 50 mg catalyst, 0.5 mL IL, 1 equiv. active methylene compound.
[b] On the basis of HPLC and product confirmation by ESI-MS/MS and NMR.
[c] Isolated yield.

Table 4. Direct catalytic synthesis of cinnamic acids by Knoevenagel–Doebner condensation.[a]



a b

Entry	R	Catalyst	Time [h]	Product [%][b]
1	4-$MeOC_6H_4$	BSA	8	92(86)[c]
2	4-$MeOC_6H_4$	PPL	8	92
3	4-$MeOC_6H_4$	lysine	12	80
4	C_6H_5	BSA	8	88
5	4-OHC_6H_4	BSA	8	89
6	4-OH-3-$MeOC_6H_3$	BSA	8	90 (83)[c]
7	4-ClC_6H_4	BSA	8	95
8	4-$NO_2C_6H_4$	BSA	8	73
9	2-C_4H_3O	BSA	72	82

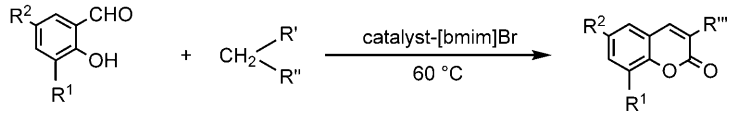
[a] *Reaction conditions:* 0.12 mmol substrate, 1 equiv. of active methylene group, 50 mg catalyst, 0.5 mL IL.
[b] On the basis of HPLC and product identification against reference standard and ESI-MS/MS.
[c] Isolated yield.

A plausible mechanism suggested the probable role of some amino acid side chain in BSA as the catalytic base[19] and IL acting as co-catalyst in above reaction. Firstly, the basic amino group removes an acidic hydrogen from active methylene compound generating a carbanion. Next, the nucleophilic attack of the carbanion on an aldehyde results in the formation of a β-hydroxy carbonyl compound. Interaction of the β-hydroxy group with ionic liquid,[20] facilitated by BSA, results in concurrent dehydration–decarboxylation–cyclization through a concerted mechanism; thus providing coumarin as the final product (Figure 2).

To probe the practical applicability of our catalytic system for aldol condensation, preparative scale reactions (1 g batch) of some substituted benzaldehydes were effectively accomplished. Furthermore, two of the isolated condensates **4b** and **5b** (Table 2) were subjected to chemoselective hydrogenation,[21] providing raspberry ketone and zingerone, respectively, in good yields (Scheme 2).

In summary we have, for the first time, uncovered the role of protein impurities in "PPL" (rather than catalytic promiscuity) for olefinic bond formations, by realizing the ability of BSA to catalyze these transformations. Accordingly, a simple, mild, inexpensive and

Table 5. One-pot, four steps cascade synthesis of coumarins *via* Knoevenagel–Doebner condensation.[a]



Entry	R^1	R^2	R'	R''	R'''	Catalyst	Time	Product [%][b]
1	H	H	COOH	COOH	H	BSA	8 h	62 (56)[c]
2	H	H	COOH	COOH	H	PPL	8 h	59
3	H	H	COOH	COOH	H	Lysine	8 h	55
4	OCH_3	H	COOEt	COOEt	COOEt	BSA	8 h	87
5	H	H	COOEt	COOEt	COOEt	BSA	12 h	94 (91)[c]
6	OCH_3	allyl	COOEt	COOEt	COOEt	BSA	12 h	72
7	H	Cl	COOEt	COOEt	COOEt	BSA	8 h	77
8	H	OH	COOH	COOH	H	BSA	8 h	45
9	H	H	$COCH_3$	COOEt	$COCH_3$	BSA	10 h	85

[a] *Reaction conditions:* 0.12 mmol substrate, 1 equiv. of active methylene group, 50 mg catalyst, 0.5 mL of IL.
[b] On the basis of HPLC and product identification by HPLC against reference standard and ESI-MS/MS.
[c] Isolated yields in parenthesis.

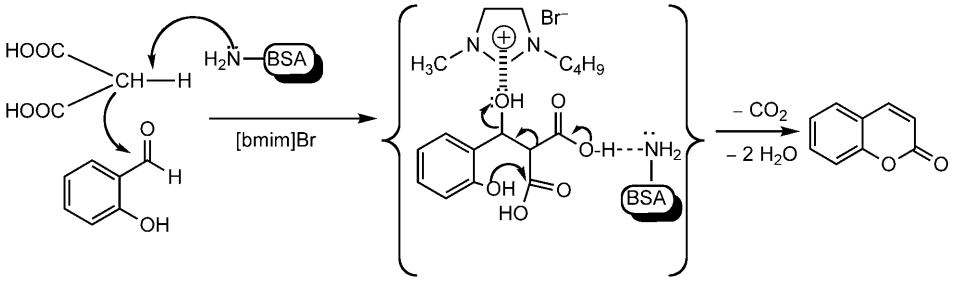
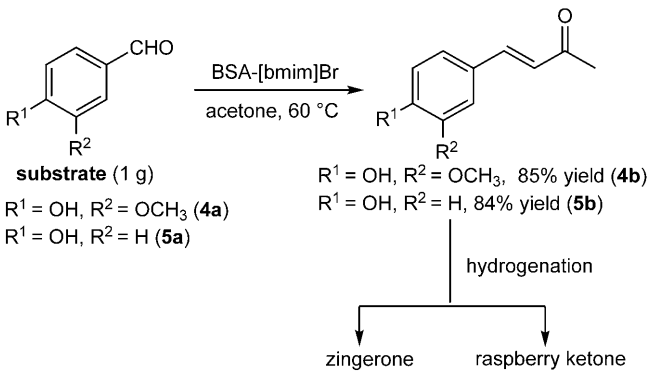


Figure 2. Proposed mechanism for the synthesis of coumarins.



Scheme 2. Preparative scale synthesis of hydroxy enones.

green synthetic methodology was developed using a synergistic combination of BSA-[bmim]Br for aldol and Knoevenagel–Doebner condensations. Other significant features of the developed protocol are: (i) it offers a promising platform for the synthesis of hydroxy enones; (ii) no dual activation of the ketone in case of chloro-, nitro- and *N,N*-dimethylbenzaldehydes; (iii) decarboxylation and/or cyclization under neutral conditions leading to the formation of cinnamic acids and coumarins in good yields. Moreover, catalyst recyclability and practical scale synthesis have been demonstrated to enhance the practical utility of process.

Experimental Section

General Procedure for the Aldol Condensations (Table 1, Table 2, Scheme 1 and Scheme 2)

Substrate (0.12 mmol), catalyst (50 mg), ketone (0.5 mL) and IL [bmim]Br (0.5 mL) were taken in a flask and the reaction mixture was incubated at 60 °C for 6 h. After the completion of the reaction, the reaction mixture was cooled, evaporated under vacuum to remove acetone and taken into ethyl acetate (4×3 mL). The combined organic layer was washed with water and dried over anhydrous Na_2SO_4 and evaporated under reduced pressure using a rotavapor

(Büchi, Switzerland). The product was analyzed by HPLC in comparison with a reference standard and further confirmed by ESI-MS/MS. For determination of isolated yield, the crude product was purified by column chromatography on silica gel with a mixture of ethyl acetate-hexane (10: 90 v/v). ^{1}H and ^{13}C NMR spectra were recorded and matched with reported values.

For, preparative scale experiments, reactions with 1 g of substrate were carried out as stated below. Substrate (1 g), BSA 2 g, ketone (35 mL) and [bmim]Br (8 mL) were taken in a flask and the reaction mixture was incubated at 60 °C for 6–8 h. After the completion of reaction, the reaction mixture was worked up as described above.

General Procedure for Knoevenagel Condensation (Table 3, Table 4 and Table 5)

Substrate (0.12 mmol), active methylene group (1 equiv.), catalyst 50 mg and IL [bmim]Br (0.5 mL) were taken in a round-bottom flask and the reaction mixture was incubated at 60 °C for 0.5–72 h. After the completion of the reaction, the reaction mixture was worked up as described above.

Fractionation of Crude "PPL"

Crude lipase from porcine pancreas was dissolved to a final concentration of 25 mg mL^{-1} in 5 mM sodium phosphate buffer at pH 7. This suspension was centrifuged for 30 min at 12000 rpm, 4 °C and the pellet was discarded.[22] Further, this solution was also filtered and then passed through a 50 kDa, 30 kDa and 10 kDa cutoff Amicon Filters (Millipore) for further fractionation (F1-F5) as described in Scheme S1, see the Supporting Information).

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